

**Stress-Dependent SUMOylation Protects Neurons from Age Related TDP-43 Toxicity with
Implications for ALS/FTD and Aging in the Mammalian Nervous System**

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Dedication

I would like to dedicate this thesis to the ~6000 Canadians who lost their battle with ALS throughout the completion of this thesis. They are a constant reminder of the urgency to better understand the causes of this disease and find effective therapeutic treatments.

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I am incredibly fortunate to be surrounded by so many individuals who supported me and want to acknowledge their contributions towards the completion of this thesis.

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Abstract

To maintain longevity, neurons must appropriately respond to and effectively recover from environmental stressors throughout their lifespan. Disruption of these stress response pathways can lead to complex neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD). The RNA binding protein TDP-43 helps facilitate appropriate stress responses through many essential roles within the nucleus. However, in ALS/FTD and conditions of prolonged stress, TDP-43 can be found aberrantly mislocalized forming large cytoplasmic aggregates causing neurotoxicity. It is critical to determine the molecular mechanisms natively regulating TDP-43 upon stress to uncover pathways that protect neurons from neurodegeneration. Covalent conjugation of SUMOs through SUMOylation has long been characterized to occur in response to cellular stress. Due to the strong overlap between roles of TDP-43 and those regulated by SUMOylation, we hypothesized that TDP-43 is likely a substrate of SUMOylation. We found that TDP-43 is SUMOylated by SUMO2/3 in a stress dependent manner at lysine 408 (K408) and is subsequently cleared through the ubiquitin-proteasome pathway upon recovery. To determine the consequences of blocking SUMOylation of TDP-43, we generated a knock-in mouse line with a TDP-43 K408R point mutation thereby preventing SUMOylation. In primary neuron cultures, we found that blocking TDP-43 SUMOylation impairs the ability for neurons to recover from stress leading to the formation of nuclear TDP-43 foci. We posited that aging may act as a stressor *in vivo* to elicit stress-dependent phenotypes and observed that TDP-43 K408R mice develop age-related cognitive impairment and motor neuron pathology. In human temporal lobe samples, we found that global SUMOylation is significantly upregulated during aging suggesting potentially important roles for SUMOylation beyond TDP-43 in protecting neurons in elderly individuals. Due to this global response, we question whether other

targets of SUMOylation may be involved in protecting different neuron populations during aging. However, uncovering roles for SUMOylation *in vivo* has been a longstanding challenge due to lack of effective tools to directly compare SUMO paralogs and unbiasedly identify native substrates. Thus, we generated a His₆-HA-SUMO2 knock-in mouse to complement a previously established His₆-HA-SUMO1 model and demonstrated using whole brain imaging and immunoprecipitation coupled with mass spectrometry that these models facilitate the direct comparison of SUMO1 and SUMO2 distribution and paralog-specific substrates *in vivo*. These models will facilitate future aging studies to uncover how global SUMOylation and specific substrates maintain neuronal longevity in health and disease.

Table of Contents

DEDICATION.....	II
ACKNOWLEDGMENTS.....	III
FUNDING.....	V
ABSTRACT.....	VI
TABLE OF CONTENTS.....	VIII
LIST OF ABBREVIATIONS.....	XIII
LIST OF MANUSCRIPTS.....	XXI
CHAPTER 1: INTRODUCTION.....	1
1.1 OVERVIEW OF TDP-43 PROTEINOPATHIES	1
1.1.1 Overview of Amyotrophic Lateral Sclerosis (ALS)	2
Clinical Manifestation and Diagnosis.....	3
Pathology	5
Genetics	7
Epidemiology and Risk Factors.....	10
Intervention.....	12
1.1.2 Overview of Frontotemporal Dementia (FTD).....	13
Clinical Manifestation and Diagnosis.....	14
Pathology	17
Genetics	18
Epidemiology and Risk Factors.....	20
Intervention.....	21
1.1.3 Limbic-predominant Age-associated TDP-43 Encephalopathy (LATE)	21
1.1.4 Related conditions associated with TDP-43 proteinopathy	22
1.2 CELLULAR STRESS RESPONSE	23
1.2.1 Key pathways and molecular complexes.....	23
1.2.2 Association with ALS/FTD and TDP-43 pathogenesis	24
1.3 TAR DNA BINDING PROTEIN 43KDA (TARDBP/TDP-43).....	26
1.3.1 Structure, Isoforms, and Proteoforms	27
<i>Figure 1. The structure and composition of full length TDP-43 and various fragments.</i>	29
1.3.2 Cellular and Molecular Functions.....	30
1.3.3 Regulation	33
1.3.4 Post Translational Modifications	35
1.4 TDP-43 PATHOGENESIS	37
<i>Figure 2. A proposed model of TDP-43 pathogenesis.</i>	38
1.4.1 Aggregation.....	39
1.4.2 Mislocalization.....	41
1.4.3 Potential Mechanisms Initiating Pathogenesis.....	44

1.5	SUMOYLATION	46
1.5.1	Molecular Functions	48
1.5.2	Roles in the Central Nervous System	49
1.5.3	Links to Aging and Neurodegeneration	51
1.6	Statement of Research Problem, Rationale, and Objectives	52
CHAPTER 2: A STRESS RESPONSIVE TDP-43 SUMOYLATION PROGRAM PRESERVES NEURONAL FUNCTION		55
2.1	STATEMENT OF AUTHOR CONTRIBUTIONS	56
2.2	ABSTRACT.....	58
2.3	INTRODUCTION	59
2.4	RESULTS.....	64
	TDP-43 is SUMOylated in the nucleus in a context specific manner	64
	<i>Fig. 1: SUMOylation dynamically regulates nuclear TDP-43 in a stress-responsive manner.</i>	66
	SUMOylation is an early event in response to stress and helps clear TDP-43 through the ubiquitin proteasome system during recovery	68
	Stress-dependent TDP-43 SUMOylation is mediated by select E3 SUMO ligases	69
	TDP-43 SUMOylation occurs in a conserved region of the C-terminal domain	70
	<i>Fig. 2: TDP-43 is SUMOylated at K408 in a conserved region of the C-terminal domain.</i>	73
	TDP-43 SUMOylation at K408 supports cellular stress response and recovery	74
	<i>Fig. 3: Blocking TDP-43 SUMOylation at conserved K408 impairs the cellular stress response.</i>	76
	Blocking TDP-43 SUMOylation in vivo leads to sex-specific social and cognitive deficits	78
	<i>Fig. 4: Blocking TDP-43 SUMOylation in vivo leads to sex-specific social and cognitive impairment.</i>	80
	TDP-43 ^{K408R} mice present with distinct features of TDP-43 pathology related to ALS and FTD	82
	<i>Fig. 5: Male TDP-43^{K408R} mice present with age-specific features of ALS.</i>	84
	Aberrant SUMOylation is a feature of human aging and disease	85
	<i>Fig. 6: SUMOylation correlates with aging and is enriched in the prefrontal cortex of ALS/FTD patients.</i>	86
2.5	DISCUSSION.....	88
2.6	MATERIALS AND METHODS.....	93
	Materials Availability.....	93
	Data and Code Availability	93
	Cloning of pEGFP-TDP-43 plasmid and variants	93
	Cell Culture.....	94
	Generation of pL302-HA-SUMO2 stable HEK293T cell line	94
	GFP-trap SUMOylation assay	94
	Endogenous SUMOylation assay	96
	Serial protein extraction from cell culture	97
	Western blot analysis	97
	E3 SUMO Ligase Screen.....	98
	Conservation analysis of TDP-43 K408.....	98
	TDP-43 ^{K408R} mouse line generation.....	99
	Mouse husbandry	99
	Mouse wellness monitoring.....	100
	Sex and age of experimental mice	100

TDP-43 ^{K408R} genotyping.....	101
Primary cortical neuron cultures.....	102
Murine Primary Neuron Culture Maintenance.....	102
Immunofluorescence in primary cortical neurons.....	103
Proximity ligation assay in primary cortical neurons.....	103
Behaviour testing general information.....	105
Developmental testing.....	105
Beam break.....	106
Digigait.....	106
Fear conditioning.....	107
Grip strength.....	107
Hanging wire.....	108
Light/dark box.....	108
Marble burying.....	109
Nest building.....	109
Open field.....	109
Rotarod.....	110
Spontaneous Y-maze.....	110
Tail suspension.....	110
Three chamber sociability.....	111
Tube test of social dominance.....	111
Collection and preparation of mouse tissue.....	112
Immunofluorescence analysis in mouse tissue.....	112
Neuromuscular junction analysis.....	113
SUMOylation analysis in human brain tissue.....	114
Collection and preparation of FFPE human brain tissue.....	115
Proximity ligation assay in human FFPE tissue.....	115
Statistical Analyses.....	116
2.7 ACKNOWLEDGMENTS.....	117
2.8 SUPPLEMENTARY INFORMATION.....	118
<i>Fig. S1: Characterizing stress responsive TDP-43 SUMOylation.</i>	119
<i>Fig. S2: Generation of the TDP-43^{K408R} mouse line.</i>	120
<i>Fig. S3: Characterizing the stress response in TDP-43^{K408R} primary cortical neuron cultures.</i>	122
<i>Fig. S4: General wellness of TDP-43^{K408R} mice during development and aging.</i>	124
<i>Fig. S6: Characterizing the motor behaviour of TDP-43^{K408R} mice.</i>	128
<i>Fig. S7: Characterizing the activity of TDP-43^{K408R} mice.</i>	129
<i>Fig. S8: Characterizing the molecular and histological effects of blocking TDP-43 SUMOylation in vivo.</i>	131
<i>Fig. S9: Optimization of proximity ligation assay in human tissue.</i>	132
<i>Table S1: K408 Conservation Analysis</i>	133
<i>Table S2: Behaviour Analysis</i>	134
<i>Table S3: Patient Information</i>	137
<i>Table S4: Reagents</i>	143
CHAPTER 3: CHARACTERIZING THE DIFFERENTIAL DISTRIBUTION AND TARGETS OF SUMO1 AND SUMO2 IN THE MOUSE BRAIN	144
3.1 STATEMENT OF AUTHOR CONTRIBUTIONS	145
3.2 ABSTRACT.....	147

3.3	INTRODUCTION.....	147
3.4	RESULTS.....	152
	Generation of (His ₆ -)HA-Sumo2 mouse lines.	152
	His ₆ -HA-Sumo2 enables the comparison of endogenous Sumo2 levels in the CNS.	152
	<i>Figure 1: HA-epitope knock-in enables direct comparisons of differential levels of Sumo1 and Sumo2 and their conjugates in the murine central nervous system.</i>	156
	Regional analysis of Sumo1 and Sumo2 throughout the mouse brain	157
	<i>Figure 2: Whole brain imaging reveals the topographical distribution of Sumo paralogs.</i>	160
	HA-Sumo2 localizes to synapses in vivo.	161
	<i>Figure 3: Extra-nuclear localization of Sumo2 in neurons of the hippocampal CA3 region.</i>	163
	<i>Figure 4: Sumo2 localizes at synapses in vivo.</i>	165
	HA-Sumo mice reveal convergent and contrasting neuronal Sumo1 and Sumo2 substrates.	166
	<i>Figure 5: Neuronal Sumo2 has shared and distinct substrates compared to Sumo1 in vivo.</i>	169
	Subcellular localization of Sumo interactions in neurons	170
	<i>Figure 6: Established and newly identified Sumo2 substrates are present both in nuclear and extranuclear compartments in neurons.</i>	173
3.5	DISCUSSION.....	174
3.6	MATERIALS AND METHODS.....	182
	STAR METHODS.....	182
	Resource Availability.....	182
	Experimental Model and Subject Details	182
	Method Details	184
	His ₆ -HA-Sumo2 and HA-Sumo2 Mouse Generation.....	184
	Mouse Genotyping.....	186
	Biochemical Analysis of Adult Mouse Brain and Spinal Cord	187
	Real Time Quantitative PCR (RT-qPCR) analysis	188
	Brain preparation for whole brain clearing and HA immunolabeling.	189
	Whole mouse brain processing, staining, and imaging	189
	HA Immunoprecipitation from mouse brain lysates.....	190
	Sample Preparation for Mass Spectrometry	191
	Protein Identification by LC-MS/MS	191
	Gene Ontology analysis.....	192
	Immunohistochemistry of Mouse Brain	192
	Primary Cortical Neuron Cultures.....	193
	Immunofluorescence in Primary Cortical Neurons.....	194
	Proximity Ligation Assay in Primary Cortical Neurons.....	195
	Tissue Lysis.....	196
	Subcellular fractionation.....	196
	Western blot analysis	196
	Confocal Imaging and Image analysis.....	197
	Quantification and Statistical Analysis.....	198
3.7	ACKNOWLEDGMENTS.....	199
3.8	SUPPLEMENTARY INFORMATION.....	200
	<i>Figure S1: Heterozygous His₆-HA-Sumo2 and His₆-HA-Sumo1 mice exhibit normal Sumo levels. Related to Figure 1.</i>	201
	<i>Figure S2: Detailed examination of regional anatomical Sumo paralog distribution. Related to Figure 2.</i>	203

<i>Figure S3: Sumo1 and Sumo2 share similar and distinct anatomical locations and subcellular compartments. Related to Figure 2.</i>	204
<i>Figure S4: Differential levels and sub-cellular distribution of Sumo1 and Sumo2 conjugates in whole mouse brain fractions. Related to Figure 3.</i>	206
<i>Figure S5: Imaris-based representation of the synaptic Sumo2 co-localization with Shank2 and Synapsin1. Related to Figure 4.</i>	208
<i>Figure S6: Neuronal Sumo1 has shared and distinct substrates compared to Sumo2 in vivo. Related to Figure 5.</i>	209
<i>Figure S7: Localization of Sumo2 to extranuclear compartments and proof of PLA assay specificity. Related to Figure 6.</i>	211
<i>Table 1: Whole Brain Imaging Analysis – Related to Figure 2</i>	212
<i>Table 2: Mass Spectrometry Analysis – Related to Figure 5</i>	213
<i>Table 3: Antibody List – Related to STAR Methods</i>	214
CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS	216
4.1 SUMMARY	216
4.2 DISCUSSION OF KEY FINDINGS	219
4.3 FUTURE DIRECTIONS	230
APPENDIX I	235
REFERENCES CITED	235
APPENDIX II	294
PERMISSION TO REPRINT PUBLISHED MANUSCRIPTS	294
<i>Suk et al. (2024) – BioRxiv</i>	294
<i>Suk et al. (2023) – iScience</i>	295
<i>Suk and Rousseaux (2020) – Molecular Neurodegeneration</i>	296
<i>Geertsma et al. (2022) – Human Molecular Genetics</i>	297
<i>Van Bruggen et al. (2023) – eNeuro</i>	298
APPENDIX III	299
APPENDED ARTICLES	299
<i>Suk and Rousseaux (2020) – Molecular Neurodegeneration</i>	299
<i>Geertsma et al. (2022) – Human Molecular Genetics</i>	316
<i>Van Bruggen et al. (2023) – eNeuro</i>	333

List of Abbreviations

ACC	Anterior Cingulate Cortex
AChR	Acetylcholine Receptor
AD	Alzheimer's Disease
ALIX	ALG-2 Interacting Protein X
ALS	Amyotrophic Lateral Sclerosis
ALSFRS-R	ALS Functional Rating Scale – Revised
ANOVA	Analysis of Variance
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
ARC	Activity Regulated Cytoskeleton Associated Protein
ASO	Antisense Oligonucleotide
ATF4	Activating Transcription Factor 4 (<i>Alias: CREB2</i>)
ATG8/12	Autophagy Related Protein 8 and 12
ATXN2	Ataxin 2
BIBD	Basophilic Inclusion Body Disease
bvFTD	Behavioural Variant Frontotemporal Dementia
C9ORF72	Chromosome 9 Open Reading Frame 72
CBX4	Chromobox 4
CBM	Cerebellum
cDNA	Complementary DNA
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGAS	Cyclic GMP-AMP Synthase

ChAT	Choline Acetyltransferase
ChIP	Chromatin Immunoprecipitation
CHOP	DNA Damage Inducible Transcript 3 (<i>Alias: DDIT3</i>)
CK1	Casine Kinase 1
CK2	Casine Kinase 2
CLIP	Cross-Linking Immunoprecipitation
CLOCK	Circadian Locomotor Output Cycles Protein Kaput
CNS	Central Nervous System
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Cerebral Spinal Fluid
CTD	C-Terminal Domain
CTE	Chronic Traumatic Encephalopathy
CTF	C-Terminal Fragment
CTSD	Cathepsin D
CTX	Cortex
DAPI	4',6-diamidino-2-phenylindol
DAT	Dopamine Transporter
DNA	Deoxyribonucleic Acid
DPR	Dipeptide Repeats
EGFP	Enhanced Green Fluorescent Protein
EGR2	Early Growth Response 2 (<i>Alias: KROX20</i>)
eIF2a	Eukaryotic Translation Initiation Factor 2a
EMG	Electromyogram

ER	Endoplasmic Reticulum
EWSR1	Ewing Sarcoma Breakpoint Region 1
fALS	Familial ALS
FI	Frontoinsular Cortex
FTD	Frontotemporal Dementia
FTD-FUS	Frontotemporal Dementia with FUS Pathology
FTD-Tau	Frontotemporal Dementia with Tau Pathology
FTD-TDP	Frontotemporal Dementia with TDP-43 Pathology
FTDP-17	Frontotemporal Dementia with Parkinsonism 17
FTLD	Frontotemporal Lobar Degeneration
FUS	Fused in Sarcoma
G3BP1	G3BP Stress Granule Assembly Factor 1
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GPCR	G-Coupled Protein Receptor
GRN	Granulin
GTF2I	General Transcription Factor 2I
GWAS	Genome Wide Association Study
HA	Hemagglutinin
HDAC2	Histone Deacetylase 2
HDGFL2	Hepatoma-Derived Growth Factor Related Protein 2
HERV	Human Endogenous Retroviruses
HHV	Herpesvirus

His₆	Histidine (x6)
HIV	Human Immunodeficiency Virus
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HRE	Hexanucleotide Repeat Expansion
HSP70	Heat Shock Protein 70kDa
HTT	Huntingtin
IDR	Intrinsically Disordered Region
IP	Immunoprecipitation
IP/MS	Immunoprecipitation/Mass Spectrometry
ISG15	ISG15 Ubiquitin Like Modifier
ISR	Integrated Stress Response
KAP1	Tripartite Motif Containing 28 (<i>Alias: TRIM28</i>)
KARS1	Lysyl-tRNA Synthetase 1
KIAA1586	E3 SUMO-Protein Ligase KIAA1586
KROX20	Early Growth Response 2 (<i>Alias: EGR2</i>)
LATE	Limbic-predominant Age-associated TDP-43 Encephalopathy
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LIG4	Ligase 4
LINE	Long-Interspersed Transposable Elements
LLPS	Liquid-Liquid Phase Separation
lncRNA	Long Non-Coding RNA
MAP2	Microtubule Associated Protein 2

MAPT	Microtubule Associated Protein Tau
MATR3	Matrin 3
miRNA	Micro RNA
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NEAT1	Nuclear Paraspeckle Assembly Transcript 1
NEDD8	NEDD8 Ubiquitin Like Modifier
NES	Nuclear Export Sequence
NeuN	Neuronal Nuclei Antigen (RNA Binding Fox-1 Homolog 3)
NfL	Neurofilament Light Chain
NHEJ	Non-Homologous End Joining
NIFID	Neuronal Intermediate Filament Inclusion Disease
NLS	Nuclear Localization Sequence
NMJ	Neuromuscular Junction
NOS	Nitric Oxide Synthase
NSMCE2	NSE2 Homolog, Smc5-Smc6 Complex SUMO Ligase
NTD	N-Terminal Domain
NUP50	Nucleoporin 50
OB	Olfactory Bulb
p62	Protein 62kDa (<i>Alias: SQSTM1</i>)
PCR	Polymerase Chain Reaction
PDSM	Phosphorylation-Dependent SUMOylation Motif

PER2	Period Circadian Regulator 2
PERK	Protein Kinase R-like ER Kinase
PET	Positron Emission Tracer
PFC	Prefrontal Cortex
PIAS	Protein Inhibitor of Activated STAT
PLA	Proximity Ligation Assay
PML	PML Nuclear Body Scaffold
PNFA	Primary Nonfluent Aphasia
PNS	Peripheral Nervous System
PPA	Primary Progressive Aphasia
PROX1	Prospero Homeobox Protein 1
PTM	Post Translational Modification
qPCR	Quantitative Polymerase Chain Reaction
RAN	Repeat Associated Non-ATG [Translation]
RANBP2	Ran Binding Protein 2
RANGAP1	Ran-GTPase Activating Protein 1
RIP	RNA Immunoprecipitation
RNA	Ribonucleic Acid
RNF212	Ring Finger Protein 212
RNF4	Ring Finger Protein 4
ROS	Reactive Oxygen Species
RRM	RNA Recognition Motif
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction

SBPV	Subparaventricular Zone
SAE1	SUMO Activating Enzyme E1
sALS	Sporadic ALS
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SC	Spinal Cord
SCH	Suprachiasmatic Nucleus
SENP	Sentrin Specific Protease
SG	Stress Granule
sgRNA	Single Guide RNA
SIM	SUMO Interacting Motif
SNP	Single Nucleotide Polymorphism
SOD1	Cu/Zn Superoxide Dismutase 1
SPM	Synaptic Plasma Membrane
SQSTM1	Sequestrosome 1 (<i>Alias: p62</i>)
STING	Stimulator of Interferon Response cGAMP Interactor 1
STMN2	Stathmin 2
STUbL	SUMO Targeted Ubiquitin Ligase
SUMO	Small Ubiquitin Like Modifier
svPPA	Semantic Variant Primary Progressive Aphasia
S-Th-B	Striatum-Thalamus-Brainstem Tract
TAF15	TATA-box Binding Protein Associated Factor 15
TAR	Transactive Response
TARDBP	TAR DNA Binding Protein 43 (Gene)

TDP-43	TAR DNA Binding Protein 43 (Protein)
TIA1	TIA1 Cytotoxic Granule Associated RNA Binding Protein
TOPORS	TOP1 Binding Arginine/Serine Rich Protein, E3 Ubiquitin Ligase
TREM106B	Transmembrane Protein 106B
TRIM28	Tripartite Motif Containing 28 (<i>Alias: KAP1</i>)
TRPV1	Transient Receptor Potential Villinoid 1 (<i>Alias: Capsaicin Receptor</i>)
TTBK1/2	Tau Tubulin Binding Kinase 1 and 2
UBA2	Ubiquitin-like modifier Activating Enzyme 2
UBC9	Ubiquitin-Conjugating Enzyme E2I (Protein)
UBE2I	Ubiquitin-Conjugating Enzyme E2I (Gene)
UMF1	Ubiquitin Fold Modifier 1
UNC13A	UNC-13 Homolog A
UPS	Ubiquitin Proteasome System
UTR	Untranslated Region
VAT1L	Vesicle Amine Transport 1-Like
VCP	Vasolin-Containing Protein
VEN	von Economo Neurons (<i>Alias: Spindle Neurons</i>)
WT	Wild Type
XRCC4	X-Ray Repair Cross Complementing 4
ZATT	Zinc Finger Protein 451 (<i>Alias: ZNF451</i>)
ZNF451	Zinc Finger Protein 451 (<i>Alias: ZATT</i>)

List of Manuscripts

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Chapter 1: Introduction

1.1 Overview of TDP-43 Proteinopathies

Neurodegenerative diseases are highly heterogeneous in nature from their clinical manifestations to their molecular underpinnings (Erkkinen et al., 2018; Wilson et al., 2023). Despite this heterogeneity, a select few proteins are recurrently observed to accumulate in the central nervous system (CNS) of those affected by neurodegenerative diseases (Dugger and Dickson, 2017; Wilson et al., 2023). The pathological accumulation of specific proteins, termed “proteinopathy”, has classically defined major neurodegenerative diseases (Bayer, 2015). These include the accumulation of β -Amyloid and Tau in Alzheimer’s Disease (Knopman et al., 2021; Lee et al., 2001), Alpha-Synuclein in Parkinson’s Disease (Goedert, 2001), Huntingtin in Huntington’s Disease (Hoffner and Djian, 2002), and TDP-43 in Amyotrophic Lateral Sclerosis (ALS) (Mackenzie and Rademakers, 2008). Each proteinopathy is thought to uniquely contribute to neurodegeneration, providing insight into different potential causes and corresponding clinical manifestations of diseases (Dugger and Dickson, 2017; Wilson et al., 2023). Increasingly, these pathologies are implicated in other neurodegenerative diseases by accumulating in distinct cell types and/or regions of the CNS, or co-existing with multiple pathologies (Robinson et al., 2023; Young et al., 2018).

TDP-43 proteinopathy is classically associated with ALS (Arai et al., 2006; Neumann et al., 2006). However, TDP-43 pathology is a major proteinopathy for distinct forms of Frontotemporal Dementia (FTD) and is characteristic of Limbic-predominant, Age-associated TDP-43 Encephalopathy (LATE) (Arai et al., 2006; Cairns et al., 2007; Kwong et al., 2007; Nag and Schneider, 2023; Nelson et al., 2019; Neumann et al., 2006). Furthermore, TDP-43 is often observed as a co-pathology in other major neurodegenerative diseases such as Alzheimer’s Disease

and Huntington's Disease and found in neurological conditions involving brain trauma including Chronic Traumatic Encephalopathy (CTE) and Stroke(Agra Almeida Quadros et al., 2024; Meneses et al., 2021; Nicks et al., 2023; Thammisetty et al., 2018).

1.1.1 Overview of Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS), also referred to as Motor Neuron(e) Disease (MND) or Lou Gehrig's Disease, is a fatal neurodegenerative disease affecting ~1000 Canadians each year with no cure and few therapeutic treatments(ALS Society of Canada, 2018; Hardiman et al., 2017). ALS is characterized by the degeneration of motor neurons in the spinal cord and motor cortex leading to progressive paralysis and death typically within 2-5 years post-diagnosis(Chiò et al., 2009; Hardiman et al., 2017). Based on the highly aggressive nature of the disease, the prevalence of ALS strongly underrepresents the risk of developing ALS throughout one's lifetime which is estimated as high as ~1:350(Kiernan et al., 2011). ALS is a complex disease with major genetic and environmental components involved in disease pathogenesis. Approximately 10% of cases occur due to inherited mutations (familial ALS, fALS) in one of >30 genes identified to cause disease(Nguyen et al., 2018; Taylor et al., 2016). The remaining ~90% occur sporadically (sporadic ALS, sALS) due to *de novo* mutations in these causative genes or through unknown mechanisms. Despite heterogenous causes of disease, nearly all patients with ALS present with TDP-43 pathology(Kwong et al., 2007; Neumann et al., 2009a, 2006). Thus, much research has focused on how various genetic and environmental mechanisms converge on TDP-43 to cause disease and whether TDP-43 may be a viable target for therapeutic intervention(Babazadeh et al., 2023; Hayes and Kalab, 2022; Scotter et al., 2015). Importantly, ALS can occur on a clinical and genetic spectrum with Frontotemporal Dementia (FTD) where patients may be diagnosed with ALS, FTD,

or ALS and FTD (referred to as “ALS/FTD”)(Burrell et al., 2016; Cividini et al., 2022; Strong et al., 2017). Approximately 13% of patients are diagnosed with behavioural variant FTD and even without a formal FTD diagnosis, up to 50% of ALS patients present with cognitive symptoms resembling aspects of FTD(Elamin et al., 2013; Phukan et al., 2012; Strong et al., 2017). Although there is a high variability in clinical presentation across the ALS/FTD spectrum, TDP-43 pathology serves as a crucial link between the two diseases(Arai et al., 2010, 2006; Cairns et al., 2007; Geser et al., 2009; Hasegawa et al., 2008; Neumann et al., 2009a, 2006).

Clinical Manifestation and Diagnosis

ALS is a fatal neurodegenerative disease that occurs in late adulthood with mean age-of-onset occurring between 40-63 years old depending on type and underlying genetics(Ingre et al., 2015). ALS is highly heterogenous in its clinical manifestations further complicated by the overlap with FTD. Clinically, the disease can be classified into three distinct subtypes, based on symptoms correlating with the onset of motor neuron neurodegeneration.

Spinal onset ALS is the most common form of ALS representing ~60% of patients(Hardiman et al., 2017). Patients with spinal onset ALS typically first experience asymmetric weakness or stiffness in their extremities (often in the legs) that progress up the limbs. This can lead to loss of fine motor control if the hands are affected, or foot drop leading to increased risk of falls if the feet are affected. Electromyogram (EMG) analysis can detect fasciculations in muscle activity that coincides with early muscle dysfunction(Liu et al., 2021). Patients may also experience muscle twitching and cramping(Caress et al., 2016). Muscle weakness is then followed by progressive muscle atrophy and paralysis restricting mobility and eventually causing immobility where patients require a wheelchair(Trail et al., 2001). Muscle paralysis continues to

progress retrograde coinciding with degeneration of motor neurons throughout the spinal cord towards the brain(Maranzano et al., 2023). As ALS progresses, denervation of the muscles in the head, neck, and face may occur requiring additional supportive care through feeding tubes and devices to assist with speech(Roche et al., 2012). Denervation of the diaphragm occurs in late stages of disease, requiring the use of ventilation to maintain oxygen flow(Dorst and Ludolph, 2019). Patients generally die of respiratory failure 2-5 years post diagnosis.

Bulbar onset ALS represents approximately one third of ALS patients. Patients typically present with dysarthria (difficulty speaking), and dysphagia (difficulty swallowing) consistent with the degeneration of upper motor neurons in the brain(Hardiman et al., 2017). Bulbar onset ALS patients may progress to develop clinical features of spinal onset ALS as lower motor neurons degenerate(Maranzano et al., 2023; Roche et al., 2012). However, diaphragm denervation, affecting respiratory function, can be affected significantly earlier in disease progression of bulbar onset ALS. Thus, bulbar onset is considered a severe form of ALS as critical processes are rapidly affected leading to death typically within 1-2 years after diagnosis(Shellikeri et al., 2017).

Respiratory onset ALS is a very rare form of ALS that affects the respiratory system first leading to difficulties breathing, common respiratory infections, and respiratory failure before the onset of motor impairment(Gautier et al., 2010). Respiratory onset ALS is particularly aggressive, typically leading to death more rapidly than bulbar onset ALS within ~1-1.5 years of diagnosis.

Cognitive impairment is increasingly recognized in ~50% of patients with ALS aligning with symptoms of FTD, regardless of whether a formal FTD diagnosis is given(Burrell et al., 2016; Cividini et al., 2022; Hardiman et al., 2017; Strong et al., 2017). Patients living with ALS may present with general changes in behaviour that may develop or progress during the duration of disease, with apathy being one of the most common behavioural changes.

There are no specific diagnostic criteria for ALS and a disease is diagnosed by clinical investigation excluding all other possible explanations for symptoms(Hardiman et al., 2017). Due to the general rarity of ALS, the high heterogeneity of symptom onset and progression, and lack of specificity with early symptoms, clinicians may often mistake these symptoms for other conditions such as Multiple Sclerosis or Muscular Dystrophy thereby delaying the diagnosis of ALS. Once diagnosed, neurologists use a Revised ALS Functional Rating Scale (ALSFRS-R) questionnaire assessing abilities to perform everyday tasks to monitor disease progression(Cedarbaum et al., 1999). This is the primary scale used in clinical trials to determine effectiveness of drug treatments(Bakers et al., 2022).

Pathology

ALS is characterized by the degeneration of upper and lower motor neurons throughout the central nervous system(Hardiman et al., 2017). Motor neurons in the spinal cord (often referred to as spinal motor neurons or lower motor neurons) are large cholinergic neurons that reside specifically in the ventral horn and project towards the periphery to innervate muscles(Stifani, 2014). There are two main types of spinal motor neurons: Alpha motor neurons which innervate extrafusal fibers that generate most of the muscles contractile power; and gamma motor neurons which innervate intrafusal fibers that play a role in muscle proprioception(Friese et al., 2009; Hof et al., 2004). Alpha motor neurons are specifically vulnerable to degeneration in ALS(Lalancette-Hebert et al., 2016). The cell body of alpha motor neurons reside within the central nervous system, specifically in the ventral horn of the spinal cord and represent ~70% of all lower motor neurons(Hof et al., 2004). Alpha motor neurons function as a part of the peripheral nervous system and innervate extrafusal muscles through neuromuscular junctions (NMJs) to drive voluntary

muscle movements through the release of acetylcholine(Brull and Naguib, 2011; Hof et al., 2004). NMJ denervation is a major component of motor neuron degeneration and can drive muscular atrophy as muscles no longer receive signals from the CNS(Verma et al., 2022). Motor neurons are thought to generally degenerate through one of two mechanisms: a “dying back” mechanism where degeneration initiates at the NMJ and progresses retrograde towards the cell body, or “dying forward” in which motor neuron degeneration initiates at the cell body and progresses anterograde towards the NMJ(Dadon-Nachum et al., 2011; Eisen, 2021).

Within the brain, upper motor neurons (also known as Betz cells or pyramidal tract neurons) are selectively vulnerable in ALS and are the first to degenerate in bulbar onset disease(Braak et al., 2017; Eisen, 2021; Nolan et al., 2024). Upper motor neurons reside within layer V of the primary motor cortex and generally innervate alpha motor neurons in the spinal cord(Nolan et al., 2024). There is a highly complex relationship between the circuitry within the prefrontal cortex and the primary motor cortex to elicit signal transduction through upper motor neurons which may explain some of the links between ALS and FTD(Kandel et al., 2013; Nolan et al., 2024; Pineda et al., 2024).

A histopathological hallmark of ~97% of all cases of ALS is the presence of TDP-43 pathology(Hardiman et al., 2017; Kiernan et al., 2011). Characteristically, TDP-43 pathology involves the nuclear-to-cytoplasmic mislocalization and subsequent aggregation of TDP-43 (see **Chapter 1.4** for characteristics of TDP-43 pathology)(Arai et al., 2006; Hasegawa et al., 2008; Kwong et al., 2007; Neumann et al., 2006; Suk and Rousseaux, 2020). The rare exceptions encompassing the remaining ~3% of ALS cases generally occur in genetic cases caused by mutations in *FUS* or *SOD1*, in which cases FUS and SOD1 are found to be aggregated, respectively(Farrawell et al., 2015; Hergesheimer et al., 2019; Kwiatkowski et al., 2009;

Mackenzie et al., 2007; Vance et al., 2009). Some research has suggested that in very rare instances, TDP-43 aggregation can be observed as a co-pathology in *SOD1* ALS cases (Jeon et al., 2018; Tan et al., 2007). TDP-43 aggregates can be observed throughout the central nervous system in ALS patients (Arai et al., 2006; Hasegawa et al., 2008; Kwong et al., 2007; Neumann et al., 2006). However, generally pathology is most prominent in affected regions and affected cells involved in ALS correlating to disease progression, specifically the primary motor cortex, spinal cord, and prefrontal cortex with cognitive impairment/FTD (Kawakami et al., 2019).

Outside of the brain and spinal cord, TDP-43 pathology can sometimes be observed in fibroblasts and muscle biopsies from ALS patients (Cykowski et al., 2018; Mori et al., 2019; Pattle et al., 2023; Riancho et al., 2020; Rubio et al., 2022; Sorarú et al., 2010; Suzuki et al., 2010). TDP-43 pathology has also been observed in axons of motor neurons within muscle fiber bundles from biopsies (Riva et al., 2022). Mild elevation TDP-43 has been observed within the cerebral spinal fluid (CSF) of ALS patients (Steinacker et al., 2008). However, as a biomarker, elevated Neurofilament Light Chain (NfL) levels in the CSF occurs pre-symptomatically, correlates with disease progression, and has recently been used as a primary readout of drug efficacy for recent clinical trials (Benatar et al., 2023, 2018; Bjornevik et al., 2021; Khalil et al., 2018).

Genetics

Approximately 10% of ALS cases are due to inherited mutations causing familial forms of ALS (fALS) (Ghasemi and Brown, 2018; Goutman et al., 2022; Hardiman et al., 2017; Kiernan et al., 2011; Nguyen et al., 2018). Typically, fALS is inherited in an autosomal dominant pattern, but can be inherited in autosomal recessive or X-linked patterns depending on the gene(s) implicated (Ghasemi and Brown, 2018). To date, over 40 genes have been identified which cause

or confer a risk of ALS explaining around 75% of all fALS cases(Akçimen et al., 2023). The remaining 90% of ALS cases occur sporadically (sALS) in which 25% of these sALS cases are attributed to *de novo* mutations in known causative genes. Twin studies have found that fALS has a heritability of ~60-86% and sALS has a heritability between 38-78% indicating a strong genetic component to the disease(Al-Chalabi et al., 2010).

The most common genetic cause of ALS is due to a hexanucleotide repeat expansion (HRE) within intron 1 of *Chromosome 9 Open Reading Frame 72 (C9ORF72)* causing roughly 40% of fALS and 7% of sALS cases from European ancestry, however, *C9ORF72* mutations are quite rare in Asian populations(Akçimen et al., 2023; DeJesus-Hernandez et al., 2011; Majounie et al., 2012; Renton et al., 2011). Healthy individuals generally harbour ~2-30 repeats in intron 1 of *C9ORF72*, whereas patients with ALS can have between 600-1000 repeats or more on average(DeJesus-Hernandez et al., 2011; Majounie et al., 2012). ALS caused by *C9ORF72* mutation is inherited in an autosomal dominant pattern, but recent evidence has suggested that these mutations may have partial penetrance as repeat expansions have been observed in unaffected individuals or in patients diagnosed with other neurodegenerative diseases such as parkinsonism or Huntington's Disease-phenocopy(Bourinaris and Houlden, 2018; Kaivola et al., 2019; Murphy et al., 2017). Additionally, as HRE of *C9ORF72* is highly unstable, somatic mosaicism may contribute to partial penetrance, disease heterogeneity, and cell type vulnerability(Kojak et al., 2024; Murphy et al., 2017; Ryan et al., 2024; Van Mossevelde et al., 2017). Although somatic mosaicism is not well understood regarding *C9ORF72* HRE, studies have found variable repeat length in neuronal and non-neuronal tissues(Nordin et al., 2015). *C9ORF72* HRE is thought to cause toxicity through a combination of haploinsufficiency causing loss of *C9ORF72* function and gain of toxicity through the formation of toxic RNA foci and through repeat associated non-ATG (RAN) translation causing the

expression of dipeptide repeats(Ash et al., 2013; DeJesus-Hernandez et al., 2011; Donnelly et al., 2013; Gendron et al., 2013; Haeusler et al., 2014; Mori et al., 2013; Tang et al., 2020; Zu et al., 2013). There is a strong correlation between *C9ORF72* hexanucleotide repeat expansion and behavioural variant FTD, and individuals harboring *C9ORF72* mutation are often diagnosed with ALS/FTD(DeJesus-Hernandez et al., 2011; Devenney et al., 2014; Hsiung et al., 2012; Renton et al., 2011).

Mutations in *Cu/Zn Superoxide Dismutase 1 (SOD1)* are the second most common genetic cause of ALS accounting for ~12% of fALS and 1-2% of sALS cases from European ancestry(Akçimen et al., 2023; Rosen et al., 1993). In Asian populations mutations in *SOD1* are the most common genetic cause of ALS(Akçimen et al., 2023; Suzuki et al., 2022). Mutations in *SOD1* are typically inherited in an autosomal dominant pattern(Akçimen et al., 2023). Patients living with ALS due to *SOD1* mutations generally present with purely motor symptoms and are part of the small fraction of ALS cases without TDP-43 pathology, instead *SOD1* aggregates as the primary proteinopathy in these individuals(Mackenzie et al., 2007; Rosen et al., 1993).

Mutations in the RNA binding protein *Fused in Sarcoma (FUS)* cause 4% of fALS and 1% of sALS cases and can be inherited in an autosomal dominant or autosomal recessive pattern depending on the affected domain of the protein(Akçimen et al., 2023; Ghasemi and Brown, 2018; Kwiatkowski et al., 2009; Vance et al., 2009). Similarly to ALS caused by *SOD1* mutations, ALS cases caused by *FUS* mutations do not present with TDP-43 pathology, instead *FUS* is found to be mislocalized from the nucleus and aggregates in the cytoplasm. These patients present with predominantly motor symptoms with the average age of onset around 35 years of age, significantly earlier than the average age of onset for all ALS cases. It is not uncommon for mutations in *FUS*

to cause highly aggressive, juvenile onset form of ALS where symptoms begin before the age of 25 years old(Hübers et al., 2015; Xiao et al., 2024; Zou et al., 2016).

Mutations in *TAR DNA Binding Protein (TARDBP)* encoding the protein TDP-43 cause approximately 4% of fALS and 1% of sALS cases and are inherited in an autosomal dominant pattern(Akçimen et al., 2023; Benajiba et al., 2009; Conforti et al., 2011; Corrado et al., 2009; Del Bo et al., 2009; Ghasemi and Brown, 2018; Kabashi et al., 2008; Nozaki et al., 2010; Rutherford et al., 2008; Sreedharan et al., 2008; Ticozzi et al., 2011; Van Deerlin et al., 2008; Williams et al., 2009). Patients with ALS due to *TARDBP* mutations generally present with motor symptoms and may experience cognitive symptoms with or without a formal diagnosis of FTD.

Collectively, mutations in *C9ORF72*, *SOD1*, *FUS*, and *TARDBP* represent most genetic forms of ALS encompassing 60% of all fALS and roughly 10% of all sALS cases(Akçimen et al., 2023). Amongst other genes that can cause ALS, RNA binding proteins are particularly enriched including *TARDBP*, *FUS*, *hnRNPA1*, *hnRNPA2/B1*, *MATR3*, *EWSR1*, and *TAF15*(Kapeli et al., 2017). Other genes implicated in ALS often converge on common pathways such as cellular stress response, DNA damage, proteostasis, nucleocytoplasmic shuttling, and axonal transport which provides insight into key pathways implicated in disease(Akçimen et al., 2023; Ghasemi and Brown, 2018; Suzuki et al., 2022).

Epidemiology and Risk Factors

As ALS is a highly heterogenous and rare disease without clear diagnostic criteria, determining the global incidence and prevalence of disease is challenging. Most epidemiological studies have been conducted within Europe, followed by North America, then Asia, all of which show a large range in estimating the burden of disease(Longinetti and Fang, 2019). The global

prevalence of ALS based on data availability, acknowledging significant under representation of non-European, -North American, or -Asian countries, is estimated at ~4 per 100 000 individuals over a 1-year period(Wolfson et al., 2023). Based on the highly aggressive nature of ALS, prevalence rate under-represents how common ALS is in the population as most patients diagnosed with ALS will die within 2-3 years(Chiò et al., 2009). Roughly, the global incidence is estimated to be about 2 cases per 100 000 people per year(Wolfson et al., 2023). Although ALS affects both sexes, there is a slight bias towards males where the lifetime risk of developing ALS for males is ~1:350 compared to ~1:400 for females(Kiernan et al., 2011).

Gene-environment interactions are thought to be a major contributor to ALS pathogenesis. Due to the relative rarity of ALS, genome wide association studies (GWAS) are often underpowered as they require very large populations to identify genetic variants associated with disease(van Rheenen et al., 2021). However, several genetic variants, while not causative, have been associated ALS, most prominently related to the genes *UNC13A* and *ATXN2*(Becker et al., 2017; Brown et al., 2022; Elden et al., 2010; Ma et al., 2022). Single Nucleotide Polymorphisms (SNPs) at *UNC13A* have a strong genetic association with more aggressive ALS prognosis(Diekstra et al., 2012; Van Es et al., 2009). Recent studies have found that loss of TDP-43 function that occurs with TDP-43 pathology in ALS, leads to cryptic mis-splicing of *UNC13A* causing downregulation of the UNC13A protein. In individuals harbouring *UNC13A* SNPs, cryptic mis-splicing is exacerbated upon TDP-43 loss of function leading to further downregulation of UNC13A(Brown et al., 2022; Ma et al., 2022). In contrast, the intermediate repeat expansion of *ATXN2* is associated with increased risk of developing ALS(Elden et al., 2010). While healthy individuals carry <25 repeats in *ATXN2* and those with >34 repeats develop Spinocerebellar Ataxia Type 2, intermediate expansion between 31-34 repeats carries a significant risk to develop ALS

and decreased survival for disease(Elden et al., 2010; Glass et al., 2022). Furthermore, intermediate repeat expansion of *ATXN2* exacerbates TDP-43 toxicity, thus whether *ATXN2* can be targeted as a potential disease modifying therapy is being explored(Amado et al., 2024; Becker et al., 2017; Van Den Heuvel et al., 2014; Zeballos C et al., 2023).

Environmental factors play a role in ALS susceptibility. However, many epidemiological studies remain underpowered and inconclusive causing controversy about potential risk factors. Athleticism and physical activity have garnered much attention as a risk for ALS as professional sports athletes and military personnel were observed to be diagnosed with ALS more frequently(Chapman et al., 2023; Mattsson et al., 2012; McKay et al., 2021; Yu et al., 2014). Coinciding with these observations, there are proposed links between traumatic brain injury and chronic traumatic encephalopathy with risk to develop ALS(Chen et al., 2007; Franz et al., 2019; Walt et al., 2018). Exposure to heavy metals, such as lead, iron, selenium, manganese, or pesticides are correlated with increased risk of ALS(Andrew et al., 2021; Ash et al., 2019; Nagata et al., 1985; Oggiano et al., 2018). Infections from viruses such as Herpesvirus 6 (HHV-6) or Herpesvirus 8 (HHV-8), correlate with increased risk of ALS(Sola et al., 2002, 1996). Additionally, viral particles from enteroviruses have been found in the ventral horn of patients diagnosed with ALS and increased reverse transcriptase levels were observed in ALS patients suggesting potential correlation with previous retrovirus infections(Berger et al., 2000; McCormick et al., 2008).

Intervention

In Canada, there are currently two drugs approved by Health Canada to treat ALS. Riluzole, under the brand name Rilutek, is thought to function by decreasing the release of glutamate thereby reducing excitotoxic stress on motor neurons(Jafari et al., 2023). On average, Riluzole increases

survival by a median of ~6 months, specifically slowing decline through late stages of disease where swallowing and breathing become affected(Fang et al., 2018). Edaravone, under the brand name Radacava, functions by reducing reactive oxygen species that cause excess damage to cells(Cruz, 2018). Survival was found to increase by a median of ~6 months in some ALS patients, specifically slowing early progression of disease but having little effect in later stages(Brooks et al., 2022; Nourelden et al., 2023). All other treatment options available are focused on relieving symptoms of disease but are not specific to the disease itself and do not alter progression.

In 2022, AMX0035 under the brand name Albrioza was approved by Health Canada for treatment of ALS in Canada(ALS Society of Canada, 2022). However, in 2024 the results of a phase 3 clinical trial (PHEONIX Trial) found that Albrioza remained safe for use but did not show significant differences in patients over a placebo control leading to the removal of Albrioza from the market in Canada and the United States(Canada.ca, 2024).

In 2023, Tofersen under the brand name QALSODY was approved in the United States as a therapeutic treatment for ALS caused by mutations in *SOD1*(Food and Drug Administration, 2023). Tofersen is an antisense oligonucleotide (ASO) therapy that was found to be safe and effective at reducing levels of SOD1 and slowing the prognosis of ALS by improved ALSFRS-R score. The improvements were observed as a part of an open label extension during the clinical trial(Miller et al., 2022). Currently, Tofersen is accepted for accelerated approval by Health Canada and a decision is expected in 2025(ALS Society of Canada, 2024).

1.1.2 Overview of Frontotemporal Dementia (FTD)

Frontotemporal Dementia (FTD) is the most common form of early onset dementia affecting individuals less than 60 years old and is a broad classification of syndromes associated

with the dysfunction of the prefrontal cortex (PFC) and/or temporal lobes of the cerebral cortex (Grossman et al., 2023). While FTD is highly heterogeneous, generally patients living with FTD present with significant changes in behaviour, executive function, and/or speech. Patients diagnosed with FTD can be classified by symptoms such as behavioural variant FTD (bvFTD), Primary Nonfluent Aphasia (PNFA), or Semantic Primary Progressive Aphasia (svPPA), or by histopathology involving Tau (FTD-Tau), TDP-43 (FTD-TDP), or FUS (FTD-FUS). Approximately 20-50% of FTD cases are genetically inherited whereas the remaining cases occur sporadically (Olszewska et al., 2016). Over 12 genes have been identified to cause or confer risk of FTD, however most inherited cases are caused by mutations in one of three genes, *C9ORF72*, *GRN*, and *MAPT* (Greaves and Rohrer, 2019; Olszewska et al., 2016). FTD can also be caused by genes traditionally known to cause ALS such as *TARDBP* or *FUS*, highlighting the intricate relationship between the diseases (Kirola et al., 2022). Other than age, there are no conclusive risk factors for developing FTD and there are no therapeutic treatments that can modify disease course (Boxer et al., 2020; Grossman et al., 2023; Tsai and Boxer, 2016).

Clinical Manifestation and Diagnosis

FTD is a highly heterogeneous group of disorders broadly affecting one's behaviour, executive function, and/or speech depending on the brain regions affected by disease (Grossman et al., 2023). Episodic memory is generally thought to be spared in FTD, differentiating it from other major dementias such as Alzheimer's Disease (Hornberger and Piguet, 2012). Generally, patients live for 8-12 years after diagnosis of FTD, but there is a large variability in the rate of disease progression with many patients surviving longer or shorter (Kansal et al., 2016). There are three main subtypes that constitute the most prominent forms of FTD, however FTD and FTD-like

diseases are also well characterized to overlap with other neurodegenerative diseases such as ALS, Alzheimer's Disease, or Parkinsonisms (Peet et al., 2021). These subtypes can be distinguished in the early stages of disease corresponding to brain regions initially affected. However, as FTD the cortex becomes more widely affected making it difficult to differentiate between FTD subtypes or related neurodegenerative diseases.

Behavioural Variant FTD (bvFTD), formerly known as Pick's Disease, is the most common form of FTD accounting for approximately 50% of cases (Fieldhouse et al., 2021; Grossman et al., 2023; Rascovsky et al., 2011). Patients with bvFTD typically present with gradual changes in behaviour, particularly displaying symptoms of apathy, disinhibition, stereotypic behaviour, emotional blunting, lack of empathy, and changes in eating behaviours (Balachandran et al., 2021). Speech and memory are generally unaffected, particularly in early stages of disease, however significant differences in episodic memory were found in most patients with bvFTD, however not to the level observed in those diagnosed with Alzheimer's Disease (Balachandran et al., 2021; Hornberger et al., 2010).

Primary Progressive Aphasia (PPA), encompassing the nonfluent and semantic subtypes (PNFA and svPPA, respectively), is characterized by changes in speech and language processing, independent of behavioural changes observed in bvFTD (Kirshner, 2014; Vandenberghe, 2016). Patients living with PNFA can present with symptoms affecting their ability to produce speech such as anomia, agrammatism, apraxia of speech, and stuttering. Generally, patients have difficulty expressing their thoughts and ideas, although their comprehension remains intact (Knibb et al., 2009). Individuals living with svPPA present with impairment of semantic memory leading to decreased ability to effectively communicate due to aphasia, associative visual agnosia, and surface dyslexia (Suárez-González et al., 2021). Often a patient's speech remains fluent, however

incorrect words may be chosen when communicating or filled by generalizations, and words or objects may not be properly recognized during comprehension or expression. Patients living with PPA may eventually become non-verbal or non-communicative in late stages of disease.

In late stages of disease, it can become challenging to differentiate between FTD subtypes as broad degeneration of the PFC and temporal lobe can occur leading to symptoms of all subtypes of FTD(Leroy et al., 2021; Peet et al., 2021). Furthermore, patients may develop symptoms of other neurodegenerative diseases as more of the brain becomes affected such as memory loss typically observed in Alzheimer's Disease(Grossman et al., 2023; Kansal et al., 2016; Peet et al., 2021). Individuals living with late stage FTD typically succumb to the disease due to complications such as malnutrition affecting a patient's organs or ability to fight infections, aspiration pneumonia, or injuries from falls.

In very early stages of disease, many of the symptoms are not specific to FTD and may be mistaken as other conditions, so eliminating other diagnoses is often a first step to a FTD diagnosis(Bott et al., 2014). With suspicion of FTD, patients may undergo neurophysiological tests to assess reasoning, memory, or communicative skills which can help differentiate different forms of dementia, or subtypes of FTD. Additionally, neuroimaging approaches such as magnetic resonance imaging (MRI) can allow for assessment of grey matter structure throughout the brain where the prefrontal or temporal lobes may show signs of atrophy(Grossman et al., 2023; Peet et al., 2021; Whitwell, 2019). Positron emission tracer scans (PET scan) using fluorodeoxyglucose tracers can assess metabolism throughout the brain to highlight dysfunctional areas to inform of an FTD diagnosis.

Pathology

FTD is characterized broadly by atrophy of the prefrontal cortex and the temporal lobe, pathologically referred to as Frontotemporal Lobar Degeneration (FTLD)(Grossman et al., 2023). The degree of FTLD is thought to be most extreme in bvFTD cases. It was recently discovered that the main vulnerable neurons are von Economo neurons (VENs, also known as spindle neurons), which reside within layer V of the anterior cingulate cortex (ACC) and the frontoinsula (FI) cortex, particularly in the right hemisphere(Santillo et al., 2013). VENs were initially thought to be exclusive to great apes, however VENs can be found in other higher order mammals that display complex social communication such as elephants and cetaceans including whales and dolphins(Butti et al., 2009; Hakeem et al., 2009; Nimchinsky et al., 1999). While the exact roles VENs is not well characterized they are thought to play roles in sensory awareness, problem solving, and social perception, and have been implicated in Schizophrenia and Autism Spectrum Disorder(Allman et al., 2005; Brüne et al., 2010). However, degeneration of VENs is specific to FTD with all cases displaying approximately 50% loss of these cells(Kim et al., 2012; Santillo et al., 2013). Additionally, fork cells are a morphologically and functionally distinct type of neuron that also reside in layer V of the ACC and FI cortex alongside VENs that are also vulnerable to early degeneration in FTD(Kim et al., 2012; Lin et al., 2019). Recent studies based on single cell RNA sequencing from ALS/FTD patients identified non-spindle and non-fork neuron vulnerability of a Vesicle Amine Transport 1-Like (VAT1L) positive neuron in layer V of the ACC, FI, and M1 motor cortex, potentially linking ALS/FTD spectrum(Pineda et al., 2024).

Histopathologically, FTD can be differentiated into 3 subtypes based on proteinopathy. FTD-Tau is characterized by Tauopathy, the accumulation of Tau protein which has traditionally been associated with Alzheimer's Disease pathology and encompasses roughly 45% of all FTD

cases(Bahia et al., 2013; Josephs et al., 2011). Tau forms neurofibrillary tangles or Pick's bodies in bvFTD (formerly known as Pick's Disease) but is also the prominent protein found in inclusions for most patients diagnosed with PNFA(Zhang et al., 2022). Tau inclusions predominantly affect layer V neurons throughout the prefrontal cortex and temporal lobes(Bahia et al., 2013; Lin et al., 2019; Zhang et al., 2022). FTD-TDP is characterized by TDP-43 proteinopathy and occurs in roughly 45% of FTD cases, generally independent from tauopathy(Bahia et al., 2013; Josephs et al., 2011). In these cases, TDP-43 is found to aggregate prominently in layer 2/3 neurons in the prefrontal cortex and temporal lobe but has been observed in spindle neurons and fork neurons, presumably prior to their degeneration(Nana et al., 2019). TDP-43 pathology in VEN and fork neurons is primarily associated with bvFTD and svPPA and correlates with symptom severity in ALS/FTD spectrum. FTD-FUS is a rare form of FTD whereby FUS is found to accumulate in patients independent of TDP-43 or Tau pathology(Huang et al., 2010; Neumann et al., 2009b; Snowden et al., 2011). This encompasses roughly 10% of all FTD cases and can cause very rare subsets of FTD such as Neuronal Intermediate Filament Inclusion Disease (NIFID) and Basophilic Inclusion Body Disease (BIBD)(Bahia et al., 2013; Snowden et al., 2011; Yokota et al., 2008).

Genetics

Genetics play an important role in susceptibility of developing FTD. Around 20-50% of FTD cases are caused by inherited mutations whereas the remaining cases occur sporadically through unknown mechanisms or *de novo* mutations(Kirola et al., 2022; Olszewska et al., 2016). Around 60% of all inherited forms of FTD are caused by mutations in one of three genes.

The most common genetic cause of FTD is due to hexanucleotide repeat expansion of *C9ORF72*, the same mutation that can cause ALS(DeJesus-Hernandez et al., 2011; Renton et al.,

2011). Patients with C9ORF72 mutation can develop more traditional forms of FTD, particularly presenting as bvFTD or sometimes svPPA, but may also present with ALS/FTD or ALS exclusively(Hsiung et al., 2012). It remains unclear why some individuals may develop different variations of ALS and/or FTD, but incomplete penetrance or environmental factors may influence differential susceptibility(Balendra and Isaacs, 2018). Patients with FTD caused by *C9ORF72* almost exclusively present with TDP-43 pathology, however some rare cases have been found to present with Tau pathology correlating with Alzheimer's Disease-like symptoms(Balendra and Isaacs, 2018; Bieniek et al., 2013; Hsiung et al., 2012).

Mutations in the *Granulin Precursor (GRN)* gene are the second most common cause of FTD encompassing ~20-25% of inherited cases, and 10% of sporadic cases(Greaves and Rohrer, 2019; Olszewska et al., 2016). Mutations in *GRN* are inherited in an autosomal dominant pattern and cause haploinsufficiency of GRN protein(Baker et al., 2006; Cruts et al., 2006). Patients with *GRN* mutations develop pure forms of FTD, particularly presenting as bvFTD but sometimes may present as PPA without clear specificity for any subvariant, with possible concurrent Parkinsonism(Chen-Plotkin et al., 2011). FTD caused by *GRN* mutations almost exclusively presents with TDP-43 pathology.

Mutations in *Microtubule Associated Protein Tau (MAPT)* is the third most common cause of FTD encompassing 5-10% of all FTD cases and are inherited in an autosomal dominant pattern(Greaves and Rohrer, 2019; Olszewska et al., 2016). Patients living with FTD caused by *MAPT* mutations almost always develop FTD with Parkinsonism (often termed “FTDP-17”) with ~95% patients displaying symptoms of bvFTD but may also present with svPPA(Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Patients diagnosed with *MAPT* forms of FTD exclusively present with Tau pathology.

Several other genes have been implicated in causing inherited FTD cumulatively representing <5% of all FTD cases, many of which overlap with FTD/ALS spectrum or ALS alone, notably: *TARDBP*, *FUS*, and *TLA1* (Abramzon et al., 2020; Borroni et al., 2009; Hirsch-Reinshagen et al., 2017; Huey et al., 2012; Neumann et al., 2009b).

Epidemiology and Risk Factors

Only recently have efforts began collecting and analyzing data specifically on FTD instead of generalizing all cases of dementia's, thus most of the knowledge on FTD comes from a few studies from small FTD centers (Leroy et al., 2021; Onyike and Diehl-Schmid, 2013). It was estimated that the prevalence of FTD is around 15 cases per 100 000 individuals in the population, and the incidence is estimated at ~3 per 100 000 people per year (Onyike and Diehl-Schmid, 2013; Ratnavalli et al., 2002). Age is an important factor coinciding with the incidence of FTD. It is generally accepted that FTD is the most common form of dementia in individuals under 60 with most patients developing symptoms of FTD between 45 and 65 years of age (Zamboni et al., 2024). Broadly, FTD is thought to affect men and women equally, however there may be some differences when accounting for genetics or subtypes of disease (Illán-Gala et al., 2021; Pengo et al., 2022). Females with ALS had a higher prevalence of carrying *C9ORF72* mutations than males, but there was no significant difference for prevalence for females or males to develop FTD. However, females with FTD were more likely than males to carry *GRN* mutations (Curtis et al., 2017).

The only conclusive risk factor for developing FTD is having a family history of dementia (Grossman et al., 2023; Soppela et al., 2022). Recent studies have begun to correlate traumatic brain injury with an approximately 3-fold increased risk to develop FTD (Jawaid et al.,

2010). These results are of particular interest as traumatic brain injury, such as concussions, are associated with the development of Tau and/or TDP-43 pathology(Gandy et al., 2014).

Intervention

There are currently no approved therapies to treat FTD. As the mechanisms underlying FTD are inherently distinct from other forms of dementia, and different brain regions are affected, approved therapies for other forms of dementia are not effective at modifying the disease course of FTD(Tsai and Boxer, 2014). Most interventions focus on well-being and maintaining quality of life such as promoting physical activity or enjoying music(Tsai and Boxer, 2014; van 't Hooft et al., 2022). In late stages of FTD, patients will become dependent on a caregiver to assist with daily tasks.

1.1.3 Limbic-predominant Age-associated TDP-43 Encephalopathy (LATE)

Limbic-predominant Age-associated TDP-43 Encephalopathy (LATE) is a newly recognized form of dementia, thought to be the second most common neurodegenerative disease behind Alzheimer's Disease affecting ~19-39% individuals in very late stages of life (>90 years old)(Jeżewski et al., 2020; Nag and Schneider, 2023; Nelson et al., 2019). Patients present with cognitive impairment affecting episodic memory, often resembling and mistaken for dementia in Alzheimer's Disease(Butler Pagnotti et al., 2023). Neuropathologically, LATE is defined by TDP-43 proteinopathy in neurons and glia generally progressing from the amygdala to the hippocampus, and finally the midfrontal cortex(Nag and Schneider, 2023; Nelson et al., 2019). While LATE can coexist with Alzheimer's Disease in a more aggressive form of dementia,

specifically coinciding with Alzheimer's Disease pathology and tauopathy, pure forms of LATE exist without other comorbidities(Harrison et al., 2021). Several genetic risk factors have been identified, specifically with SNPs identified in genes such as *GRN* and *TREM106B* (classically linked to FTD) and in *APOE* (classically linked to Alzheimer's Disease) confer a risk for LATE(Nelson et al., 2024). Currently, there are no therapeutic treatments for LATE.

1.1.4 Related conditions associated with TDP-43 proteinopathy

Increasingly, TDP-43 pathology is recognized in an array of – predominantly neurodegenerative – conditions, highlighting the tight interconnectivity between TDP-43 and disease(De Boer et al., 2021). For example, approximately 50% of patients diagnosed with Alzheimer's Disease present with TDP-43 co-pathology alongside traditional Tau and β -Amyloid pathology(Meneses et al., 2021). TDP-43 pathology is also documented alongside neurological trauma, such as around the site of an ischemic stroke, or after concussive head trauma (Meneses et al., 2021; Nicks et al., 2023; Thammisetty et al., 2018). Furthermore, TDP-43 and Tau pathology are both characterized in patients diagnosed with chronic traumatic encephalopathy(Gandy et al., 2014; Nicks et al., 2023; Phansalkar et al., 2023). These conditions further strengthen the link between central nervous system dysfunction mediated through external factors such as genetic influence or injury and TDP-43 pathology. However, counterarguments suggest that TDP-43 pathology may be coincidental with neuronal dysfunction, hence a better understanding of the consequences of TDP-43 pathology and how it may drive, or protect, from neuronal dysfunction is critical to better understand the implications of TDP-43 pathology(Hergesheimer et al., 2019).

1.2 Cellular Stress Response

The cellular stress response is an encompassing term referring to various orchestrated, pro-survival, responses by the cell in reaction to noxious stimuli (Galluzzi et al., 2018). These stimuli can include oxidative stressors, Endoplasmic Reticulum (ER) stress, DNA damage, virus infection, unfolded proteins, nutrient starvation, chemical exposure, and heat shock responses. The cellular stress response is highly adaptive and serves to protect cells during stress allowing for reallocation of resources towards alleviating the stress, preventing excessive damage, and preparing how to recover. Prolonged or impaired stress responses often leads to activation of classic or atypical cell death pathways (Galluzzi et al., 2018; Guan et al., 2017; Wang et al., 2022). The cellular stress response is tightly linked to ALS/FTD and based on genetic, molecular, pathological, and epidemiological evidence. Additionally, TDP-43 plays active roles in various stress response pathways and prolonged cellular stress is thought to contribute to TDP-43 pathogenesis.

1.2.1 Key pathways and molecular complexes

While the cellular stress response is a systematic response involving many interconnected pathways, classically there are a few integral pathways that are generally considered. At the center of these is the integrated stress response (ISR) which is a conserved response in eukaryotes that senses various types of external stimuli and triggers a stress state within the cell (Pakos-Zebrucka et al., 2016). Central to the ISR is the serine/threonine kinase eukaryotic translation Initiation Factor 2a (eIF2a) (Pain, 1996; Pakos-Zebrucka et al., 2016; Scheuner et al., 2001). Upon stress, several ISR regulators will activate eIF2a in response to a noxious intrinsic or extrinsic stimulus, for example upon ER stress due to unfolded proteins, Protein Kinase R-like ER Kinase (PERK) signals for eIF2a activation (Berlenga et al., 1998; Perkins and Barber, 2004). Upon eIF2a

activation, Activating Transcription Factor 4 (ATF4) is translocated into the nucleus and functions as a primary transcription factor in the ISR(B'Chir et al., 2013; Karpinski et al., 1992). ATF4 in concert with other transcription factors and interacting partners will initiate transcription of genes that will aid in the resolution and recovery from the stress stimulus(B'Chir et al., 2013). For example, upregulation of heat shock proteins in response to unfolded proteins(Zhu et al., 2012).

Simultaneously, upon activation of ISR, activated eIF2a will generally signal to pause translation resulting in the formation of a stress granule (SG)(McInerney et al., 2005). Importantly, SG formation does not always require ISR activation, and ISR activation does not always signal SG formation exemplifying the complexities of cellular stress responses(Dang et al., 2006). SGs are membraneless organelles consisting of proteins and mRNA undergoing LLPS and is generally thought to assemble to protect ribosomes stalled in translation and to prepare for stress recovery(Protter and Parker, 2016). Upon activation, SGs will assemble at the sites of stalled translation, initiated by SG nucleators such as G3BP1 or TIA1(Hofmann et al., 2021). These nucleators will further promote the recruitment of other proteins, many of which are canonical RNA binding proteins containing IDRs, and mRNA. Upon recovery from stress, SGs will disassemble enabling translation to begin again.

1.2.2 Association with ALS/FTD and TDP-43 pathogenesis

There is evidence that chronic stress plays a key role in ALS/FTD and is particularly integral to motor neurons. The lifelong activation of motor neurons to signal for muscle contraction can lead to excitotoxicity(Coyle and Puttfarcken, 1993; Van Den Bosch et al., 2006). This excitotoxicity ultimately leads to the accumulation of reactive oxygen species (ROS) causing oxidative stress. Motor neurons are highly dependent on pathways capable of clearing oxidative stress

and removing ROS(Barber et al., 2006; Cookson and Shaw, 1999; Houldsworth, 2023; Onda-Ohto et al., 2023). One such protein that functions as a key antioxidant is SOD1. Overactivation, potentially through excitotoxicity, of SOD1 is thought to contribute to its misfolding causing gain of function toxicity(Kim et al., 2007). Thus, excitotoxicity may be a key player explaining motor neuron vulnerability in ALS. Pharmacological evidence supporting these theories exists as the approved ALS treatment Edaravone is designed as a free ROS scavenger, functioning to reduce oxidative stress in patients with ALS(Amekura et al., 2022). Finally, some epidemiological evidence exists suggesting that extreme physical activity such as high-level sports or military service may be a risk factor for disease(Chapman et al., 2023; Julian et al., 2021; Mattsson et al., 2012).

Activation of the ISR is observed in patients diagnosed with ALS/FTD and many ALS/FTD associated genes play roles in various pathways involved in the cellular stress response(Marlin et al., 2022). Antisense RNA produced from the *C9ORF72* HRE is sufficient to trigger the activation of the ISR(Parameswaran et al., 2023). Furthermore, DPRs can disrupt SG formation impairing a cells ability to respond and recover from stress(Keiten-Schmitz et al., 2020). Mutations in the SG nucleator protein TIA1 cause ALS/FTD with TDP-43 pathology(Hirsch-Reinshagen et al., 2017; Mackenzie et al., 2017). Intermediate CAG repeat expansion of *ATXN2* is a known genetic risk factor for ALS(Elden et al., 2010). *ATXN2* is a characterized component of SGs, but intermediate repeat expansion can lead to impaired stress response and sequestration of TDP-43(Elden et al., 2010; Nonhoff et al., 2007). Several other ALS/FTD genes, including *ANXA11*, *FUS*, *hnRNPA2/B1*, *SQSTM1*, and *VCP* are all characterized components of SGs, thus garnering much attention from the field(Fecto et al., 2011; Johnson et al., 2010; Kim et al., 2013; Markmiller et al., 2018; Nguyen et al., 2018; Topp et al., 2017; Vance et al., 2009; Wolozin and

Ivanov, 2019). Many SG components can also be observed in TDP-43 aggregates(Chen and Cohen, 2019). A prominent theory into the formation of TDP-43 aggregates is due to prolonged cellular stress causing excessive localization of TDP-43 in phase separated SGs leading to toxic phase transitions and aggregation(Asakawa et al., 2020; Gasset-Rosa et al., 2019; Guo et al., 2018; Mann et al., 2019; Ratti et al., 2020; Zhang et al., 2019). Furthermore, disruption or chronic activation of cellular stress responses are thought to sensitize cells, and particularly TDP-43, promoting hyperactive responses leading to aggregation(Barmada et al., 2010; Gasset-Rosa et al., 2019; Mann et al., 2019; Suk and Rousseaux, 2020). Thus, cellular stress responses provide extrinsic mechanisms functioning independently, or in parallel with genetic risk factors to drive ALS/FTD and TDP-43 pathogenesis.

1.3 TAR DNA Binding Protein 43kDa (TARDBP/TDP-43)

The TAR DNA Binding Protein 43kDa (TDP-43) encoded by the *TARDBP* gene is a conserved and essential DNA/RNA binding protein notoriously linked to neurodegenerative diseases, particularly ALS and FTD(Liao et al., 2022; Mackenzie and Rademakers, 2008). TDP-43 is ubiquitously expressed and predominantly localized to the nucleus but is thought to shuttle between the nucleus and the cytoplasm(Ayala et al., 2008). Within the cytoplasm TDP-43 plays several physiological roles including regulating stress-responses, protein-translation, axonal transport, and mitochondria dynamics(Alami et al., 2014; Chu et al., 2019; Colombrita et al., 2009; Fallini et al., 2012; Freibaum et al., 2010; Izumikawa et al., 2017; Majumder et al., 2016; Neelagandan et al., 2018; Štalekar et al., 2015). In a disease state, TDP-43 is found to mislocalize from the nucleus to the cytoplasm, resulting in a concurrent loss of nuclear function and gain of cytoplasmic function, and forming large protein aggregates(Suk and Rousseaux, 2020).

1.3.1 Structure, Isoforms, and Proteoforms

TDP-43 is a 414 amino acid protein related to the heterogeneous nuclear ribonucleoprotein (hnRNP) family of RNA binding proteins sharing many structural and functional similarities (Figure 1). TDP-43 is generally divided into 4 domains: The N-terminal domain (NTD), the RNA Recognition Motifs 1 and 2 (RRM1 and RRM2), and the C-terminal domain (CTD)(Buratti, 2008; Buratti and Baralle, 2001; Hergesheimer et al., 2019; Wang et al., 2016; Winton et al., 2008). Upstream of RRM1, TDP-43 contains a bipartite nuclear localization sequence (NLS) facilitating nuclear entry(Ayala et al., 2008). Within RRM2, TDP-43 contains a nuclear export sequence (NES), however the functionality of this motif has been debated as to whether it confers active export from the nucleus(Archbold et al., 2018; Ayala et al., 2008; Ederle et al., 2018; Pinarbasi et al., 2018). TDP-43 also contains 5 mitochondrial localization sequences (M1 – M5), of which M1, M3, and M5 were functionally characterized to play a role in TDP-43 localization to the mitochondria(Wang et al., 2016).

The CTD has garnered much attention in the context of ALS and neurodegeneration as nearly all the ALS and ALS/FTD causing mutations in TDP-43 occur within this domain(Kabashi et al., 2008; Van Deerlin et al., 2008). The CTD is an intrinsically disordered region (IDR) with relatively low conservation compared to other domains of TDP-43(Afroz et al., 2017; Johnson et al., 2009; Lim et al., 2016). As with other proteins containing IDRs, the CTD is characterized to enable TDP-43 to undergo liquid-liquid phase separation (LLPS)(W Michael Babinchak et al., 2019; Gopal et al., 2017). Within the CTD, there is a conserved Q/N rich domain that can transiently form an alpha-helical secondary structure which is thought to be involved in regulating TDP-43 homodimerization during LLPS(Conicella et al., 2020).

The *TARDBP* gene in humans and mice contains 6 protein coding exons and a particularly long and conserved exon 6 which contains the 3' untranslated region (UTR)(Gitcho et al., 2009; Ota et al., 2004). Alternative splicing can produce short forms of TDP-43 starting at an alternative translational start site, removing the NTD(Xiao et al., 2015). Additionally, alternatively spliced isoforms that translate C-terminally truncated forms of TDP-43 contain alternative amino acid sequences harboring a functional NES(Weskamp et al., 2020). Additionally, several studies have uncovered cryptic exons residing within the 3'UTR, now identified as exons 7, 8, and 9(Avendaño-Vázquez et al., 2012). These exons are differentially spliced in response to TDP-43 binding as a form of autoregulation affecting whether the transcripts become translated or are removed through nonsense mediated decay(Dormann et al., 2009; Herskowitz et al., 2012; Yamashita et al., 2012; Zhang et al., 2007). While the 43kDa form of TDP-43 is the prominent proteoform, shortened forms of TDP-43 at ~35kDa and ~25kDa (TDP-35 and TDP-25 respectively) have been extensively described as hallmarks of disease, particularly within the brain(Arai et al., 2010). Further truncated forms of TDP-43 at ~12kDa have also been described in disease(Dormann et al., 2009; Shenoy et al., 2019). These truncated forms of TDP-43 may be produced through several processes including alternative splicing and translation at alternative start sites or through proteolytic cleavage through various enzymes (Figure 1). These methods produce different forms of TDP-43 generally around 35kDa or 25kDa, however functional comparisons between different forms of TDP-43 at similar molecular weights has not been explored. Generally, cleaved forms of TDP-43 resulting in 35kDa and 25kDa containing the C-terminal domain, known as C-terminal Fragments (CTF-35 and CTF-25, respectively), are thought to contribute most to aggregation propensity(Dormann et al., 2009; Igaz et al., 2009; Kasu et al., 2018; Kitamura et al., 2017; Shenoy et al., 2019).

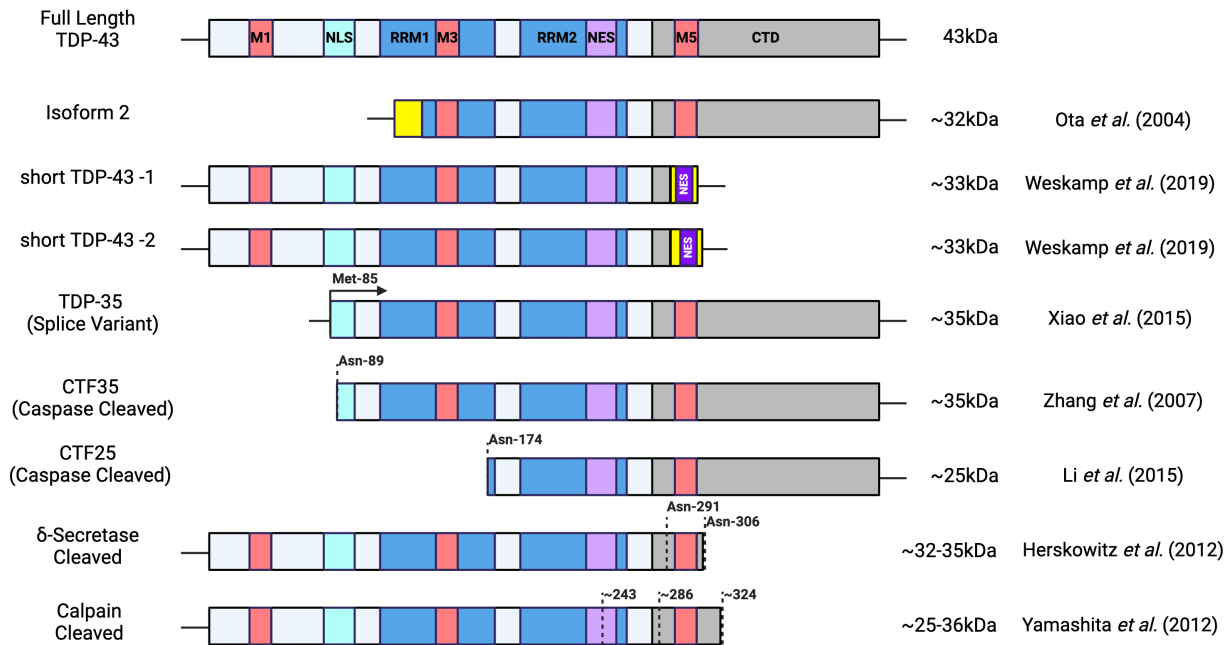


Figure 1. The structure and composition of full length TDP-43 and various fragments.

Fragments of TDP-43 can be generated through alternative splicing or various proteolytic cleavage events at different residues. The resulting fragments and remaining (or lost) features may explain their involvement in TDP-43 pathogenesis. M1,M3,M5 (Red): Mitochondria Localization Sequences; NLS (Turquoise): Bipartite Nuclear Localization Sequence; RRM1,RRM2 (Blue): RNA Recognition Motif; NES (Light Purple): Controversial Nuclear Export Signal; NES (Dark Purple): Nuclear Export Signal; CTD (Grey): C-Terminal Domain; Yellow Box: Alternate Amino Acid Sequence (N-Terminus of “Isoform 2” and C-Terminus of “Short TDP-43”); Dashed Lines: Cleavage Sites. From (Suk and Rousseaux 2020)

1.3.2 Cellular and Molecular Functions

As a predominantly nuclear localized protein, most of the characterize roles for TDP-43 occur within the nucleus and involve interactions with nucleic acids. TDP-43 was initially described as a DNA binding protein which bound the *Transactive Response* element (*TAR*) of the HIV genome(Ou et al., 1995). Later, TDP-43 was found to bind RNA and regulate splicing of the *Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)*(Buratti et al., 2004, 2001; Buratti and Baralle, 2001). Further studies involving the knockdown of TDP-43 demonstrated broad transcriptomic changes and dysregulation of alternative splicing(Polymenidou et al., 2011). Studies using RNA immunoprecipitation (RIP) or Cross-Linking Immunoprecipitation (CLIP) based approaches show that TDP-43 targets thousands of unique RNA transcripts, including mRNA, lncRNA, and miRNA(Sephton et al., 2011; Tollervey et al., 2011). Furthermore, TDP-43 preferentially, although not exclusively, binds RNA at UG-rich motifs often occurring within the 3'UTR playing a role in mRNA transcript stability(Tollervey et al., 2011). A major subset of RNA targets bound and regulated by TDP-43 are involved in synaptic activity. TDP-43 is a major repressor of cryptic exon inclusion which is currently of high interest to the field of ALS/FTD as cryptic exon inclusion of various genes has been found to drive disease or serve as potential biomarkers (See **Chapter 1.4.2**)(Jeong et al., 2017; Ling et al., 2015). In addition, TDP-43 can regulate alternate poly-adenylation as a mechanism to regulate mRNA metabolism(Arnold et al., 2024; Bryce-Smith et al., 2024; Mckeever et al., 2023; Zeng et al., 2024).

The DNA binding roles of TDP-43 are not as well characterized as its roles for RNA binding. Through Chromatin Immunoprecipitation (ChIP) studies, TDP-43 was found to bind to TG-rich motifs on DNA, albeit not exclusively(Berson et al., 2017; Kuo et al., 2009). TDP-43 is

thought to generally act as transcription factor as both an activator and a repressor to regulate gene expression(Lalmansingh et al., 2011; Morera et al., 2019; Schwenk et al., 2016). Additionally, there is evidence that TDP-43 can regulate the formation of R-loops consisting of DNA:RNA hybrids which can function as transcriptional enhancers or form during DNA replication in dividing cells(Hou et al., 2024; Niehrs and Luke, 2020; Wood et al., 2020). TDP-43 also plays a key role in regulating endogenous retroviruses and transposable elements such as Human Endogenous Retroviruses (HERVs) and Long-Interspersed Transposable Elements (LINEs) which become activated upon TDP-43 loss of function(Bilican et al., 2012; Chang and Dubnau, 2023; Li et al., 2022; E. Y. Liu et al., 2019; Pereira et al., 2018; Simula et al., 2021).

While generally thought of as a nuclear protein, TDP-43 is highly dynamic and localizes to various subcellular locales to regulate cellular function. Within the nucleus, TDP-43 localizes to various subnuclear compartments and major functional complexes. TDP-43 can co-localize with membraneless organelles such as paraspeckles and regulate their formation through alternative splicing of the long noncoding RNA (lncRNA) *Nuclear Paraspeckle Assembly Transcript 1 (NEAT1)*(Modic et al., 2018; Shelkownikova et al., 2018; Wang et al., 2020). The association with paraspeckles is demonstrated to modulate stem cell differentiation(Modic et al., 2018). TDP-43 also forms anisosomes upon proteotoxic stress, which consist of TDP-43 oligomers not bound to RNA forming a dense shell around a liquid-like core particularly consisting of heat shock proteins 70kDa (HSP70)(Yu et al., 2021). Upon oxidative stress, TDP-43 was also found to localize to liquid-like nuclear bodies, although the exact functions of these nuclear bodies remain unclear(Wang et al., 2020). TDP-43 also plays roles in shuttling mature mRNA to the cytoplasm and throughout various compartments in the cell(Ayala et al., 2008; Pinarbasi et al., 2018). Within the cytoplasm, TDP-43 plays roles in axonal and dendritic transport of mRNA(Alami et al., 2014).

Accumulating evidence that TDP-43 also plays important roles in maintaining synaptic function both at the NMJ and at synapses in other neuron cell types(Altman et al., 2021; Piol et al., 2023; Wong et al., 2021). For example, TDP-43 can regulate synaptic vesicle transport and local translation of synaptic proteins(Altman et al., 2021; Chu et al., 2019; Ishiguro et al., 2016; Majumder et al., 2016). Furthermore, loss of TDP-43 function leads to synapse loss and a decrease in dendritic complexity(Herzog et al., 2020).

TDP-43 plays important roles in the cellular stress response, and particularly for the formation of SGs. Within the nucleus TDP-43 binds and regulates alternative splicing of *G3BP1* transcripts(Khalfallah et al., 2018; McDonald et al., 2011; Sidibé et al., 2021). Upon TDP-43 loss of function, via downregulation or mislocalization, alternative splicing of *G3BP1* transcripts become disrupted leading to significant impairment of G3BP1 stress granules. TDP-43 is also suggested to localize to stress granules early in their formation but will exit stress granules shortly afterwards forming separate TDP-43 granules adjacent to SGs(Herzog et al., 2020; Lee et al., 2021; Mann et al., 2019; Mark Fang et al., 2019; Markmiller et al., 2018; Yan et al., 2024). Beyond SGs, TDP-43 will also localize to other phase separated structures in response to stress such as nuclear stress bodies, paraspeckles, P-bodies, and PML bodies however many of these functions remain elusive(Gasset-Rosa et al., 2019; Jung et al., 2023; Modic et al., 2018; Wang et al., 2020). TDP-43 is a known transcriptional regulator of the *CHOP* gene which is a regulator of apoptosis and a target and interactor of ATF4 in response to stress(Suzuki and Matsuoka, 2012). In response to DNA damage, TDP-43 helps recruit the LIG4-XRCC4 complex to sites of double stranded breaks(Guerrero et al., 2019; Kawaguchi et al., 2020; Mitra et al., 2019). Thus, TDP-43 is a stress responsive protein playing a variety of roles in various stress response pathways.

1.3.3 Regulation

Based on the variety of roles that TDP-43 plays in cells, the wide number of RNA and DNA targets, and the dynamic changes in localization, it is not surprising that tight regulation of TDP-43 must be maintained to prevent TDP-43 associated toxicity.

Models which modulate TDP-43 levels display a high range of toxicity and phenotypes based on the degree of under- or overexpression. Knockout of *Tardbp* in mice results in embryonic lethality whereas overexpression in mice causes a highly aggressive ALS-like phenotype including progressive paralysis and early lethality by 1 month of age (Kraemer et al., 2010; Wegorzewska et al., 2009; Wils et al., 2010). Mild knockdown or overexpression of *Tardbp* in mice also lead to transcriptomic and behavioural changes reminiscent of ALS/FTD indicating that mild changes in protein levels are toxic (Milstead et al., 2023; Yang et al., 2022). However, recent evidence suggests that overexpression of TDP-43, although effective at inducing TDP-43 pathology in cell models, does not represent transcriptomic profiles observed in ALS/FTD (Carmen-Orozco et al., 2024). Thus, TDP-43 loss of function may be an important driver of disease.

TDP-43 levels are highly regulated on all levels including transcriptionally, post-transcriptionally, translationally, and post-translationally. Additionally, cells can effectively compensate for changes in *TARDBP* expression. Loss of one *TARDBP* allele does not alter TDP-43 protein levels compared to cells with two copies, as cells can compensate for this loss (Sephton et al., 2010). In mouse models with mild over expression, *Tardbp* transcripts overexpressed by approximately 8-fold achieve a 10-20% increase in protein (Yang et al., 2022). A major regulator of TDP-43 levels is through autoregulation of its own transcript (Avendaño-Vázquez et al., 2012; Ayala et al., 2011; Dykstra et al., 2024). As TDP-43 levels rise, TDP-43 increasingly recognizes its own transcript causing downregulation of *TARDBP* through nonsense mediated decay.

Conversely, upon TDP-43 loss of function (via downregulation or mislocalization), alternative polyadenylation occurs on *TARDBP* due to lack of TDP-43 binding leading to increased translation(Ayala et al., 2011). On the protein level, TDP-43 cleavage, Ubiquitin-Proteasome System (UPS), endo-lysosomal pathways, extracellular vesicles, and autophagy have all been demonstrated to be involved in regulating TDP-43 levels(Chatterjee et al., 2024; Feiler et al., 2015; Iguchi et al., 2016; Leibiger et al., 2018; G. Liu et al., 2019; Liu et al., 2017; Scotter et al., 2014; Wang et al., 2010).

Subcellular localization also plays a critical role in TDP-43 functions and maintenance of its own levels. TDP-43 is actively localized to the nucleus through its nuclear localization sequence(Ayala et al., 2008). Importin Beta (KPNB1) is a key importin that transports TDP-43 into the nucleus and upon disruption can cause TDP-43 to accumulate in the cytoplasm(Aizawa et al., 2019; Khalil et al., 2022; Pinarbasi et al., 2018). The export of TDP-43 from the nucleus however remains controversial. While TDP-43 contains a nuclear export sequence, mutagenesis of this sequence does not alter TDP-43 export suggesting that TDP-43 may be passively exported from the nucleus(Pinarbasi et al., 2018). However, knockout of several exportins demonstrated altered localization of TDP-43 suggesting that active nuclear export may occur(Archbold et al., 2018; Ederle et al., 2018). Alternate hypotheses suggest that TDP-43 may piggyback with other protein interactors or binds to mRNA being translocated(Pinarbasi et al., 2018). The localization of TDP-43 is also highly dynamic and can shift under different cellular conditions. Particularly, TDP-43 physiologically localizes to the cytoplasm under stressed conditions to play a role in the cellular stress response(Khalfallah et al., 2018; Lee et al., 2021). Thus, the localization, despite being highly regulated, is also dynamic in response to cellular conditions. This adds additional

layers of complexity as cells are required to maintain and alter TDP-43 function dependent on cellular conditions.

1.3.4 Post Translational Modifications

Post translational modifications play key roles in regulating proteins, enabling high level of temporal control over the functions and localization(Buratti, 2018). Aberrant PTM is also observed as hallmarks of many diseases including TDP-43 in ALS/FTD and related proteinopathies. A variety of PTMs are characterized to modify TDP-43 with many of the functional consequences being actively uncovered.

Phosphorylation of TDP-43 is the most notorious of PTMs due to its association with TDP-43 proteinopathies. Phosphorylation at Serine (S) 403 and S404 (“S403/404”) and S409 and S410 (“S409/410”) are hallmarks of TDP-43 aggregates in disease(Neumann et al., 2009a). While classically considered to be a toxic modification, evidence has demonstrated potentially physiological roles for phosphorylation at these residues, particularly for regulating solubility and LLPS. Casein Kinase 1 (CK1) and Casein Kinase 2 (CK2) are thought to be the primary kinases triggering phosphorylation of TDP-43 at S403/404 and S409/410 and are upregulated in disease(Carlomagno et al., 2014; Ko et al., 2024; Krach et al., 2018). Targeting CK1 or CK2 has gained attention as a potential therapeutic intervention for ALS. Small molecule inhibition of CK1 can decrease TDP-43 aggregation and prion-like spread in cell and Drosophila models of ALS and TDP-43 toxicity(Cuevas et al., 2024; Martínez-González et al., 2020; Salado et al., 2014). Inhibiting Tau Tubulin Binding Kinase 1 and 2 (TTBK1/2), which also selectively phosphorylate TDP-43 at S403/404 and S409/410, results in reduced aggregate formation and toxicity in cell models of disease(Liachko et al., 2014; Martinez-Gonzalez et al., 2023; Taylor et al., 2018). Although most studies focus on phosphorylation as it relates to TDP-43 pathology, few studies

have investigated the normal regulatory roles of phosphorylation. For instance, TDP-43 is also phosphorylated at T153 and T155 in the RRM altering the subnuclear localization of TDP-43 during heat shock response(Li et al., 2017).

Ubiquitination is another hallmark of TDP-43 aggregates in disease. TDP-43 can be ubiquitinated and polyubiquitinated at various lysine (K) residues, particularly within the RRM and NTD(Hans et al., 2018, 2014). Ubiquitination is an important regulator of TDP-43 as the UPS system is thought to be one of the primary mechanisms of TDP-43 clearance(Wang et al., 2010). Acetylation of TDP-43 also occurs in disease and can be found in TDP-43 aggregates(Cohen et al., 2015). Mutation of K145 to glutamine (Q) serves as an acetylation mimetic (K145Q) and causes toxicity in mouse models leading to cognitive dysfunction and significant alterations in TDP-43 regulation of mRNA(J. C. Necarsulmer et al., 2023). It is hypothesized that acetylation at this site blocks the binding of nucleic acid within the RRM leading to loss of function. Additionally, acetylation at K136 can alter TDP-43 solubility and drive aggregation while acetylation at K84 induces mislocalization(Garcia Morato et al., 2022). Together, acetylation is generally thought to be a toxic modification.

Recently few studies have implicated the covalent conjugation of Small Ubiquitin Like Modifiers (SUMOs) to regulate TDP-43 function, however the evidence remains conflicting. These reports suggest that TDP-43 is targeted by SUMO1 likely at K136(Maraschi et al., 2021; Marino et al., 2023; Maurel et al., 2020). Mutation of K136 to block suspected SUMOylation (K136R) leads to the formation of insoluble aggregates and alterations in splicing targets of TDP-43. Additionally blocking SUMOylation through targeting the SUMO1 pathway or K136R mutation leads to TDP-43 mislocalization and aggregate formation(Marino et al., 2023; Maurel et al., 2020). However, direct evidence demonstrating that K136R lead to a loss of SUMOylation was

not demonstrated, thus it remains challenging to infer direct effects of SUMO1 on TDP-43(Maraschi et al., 2021).

Together, PTMs play a key role in regulating TDP-43 function and have garnered much attention into how they may be implicated in TDP-43 and serve as therapeutic targets(Buratti, 2018). However, much less is known about how, or whether, these modifications under physiological conditions may promote TDP-43 pathogenesis, or whether they occur concurrently or in response to disease onset.

1.4 TDP-43 Pathogenesis

TDP-43 pathology is characterized by the presence of large cytoplasmic aggregates of consisting predominantly of TDP-43(Kwong et al., 2007). However, these inclusions also accumulate with a variety of other proteins, post translational modifications, and nucleic acids, most notably: Ubiquitin[ation], hyperphosphorylation, and mRNA(Arai et al., 2006; Kwong et al., 2007; Neumann et al., 2009a, 2006). The exact chronology of TDP-43 pathogenesis remains unclear, however accumulating evidence suggests that mislocalization resulting in a loss of nuclear function occurs upstream of aggregation and may be a key driver of ALS/FTD and neurodegeneration (Figure 2)(Suk and Rousseaux, 2020).

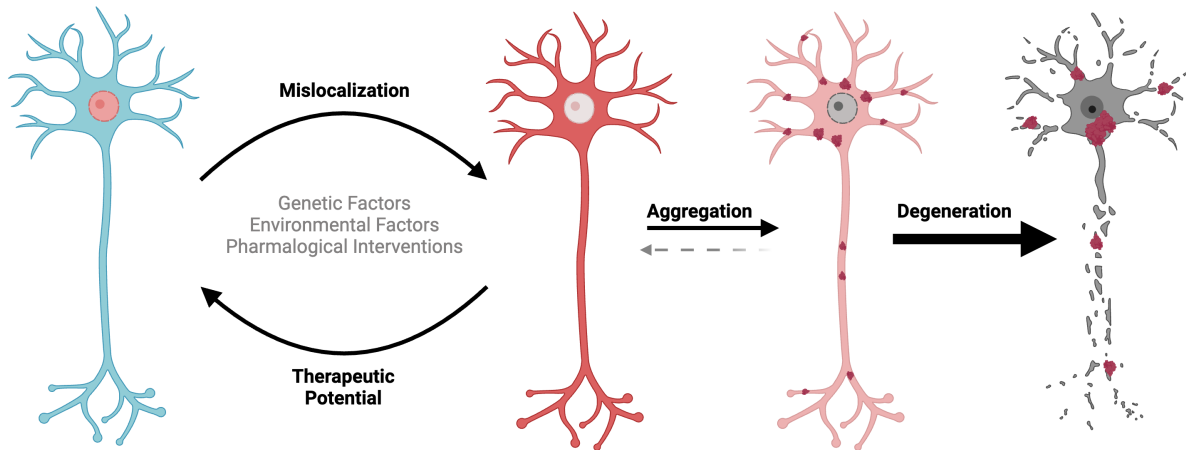


Figure 2. A proposed model of TDP-43 pathogenesis.

TDP-43 (Red) physiologically resides within the nucleus but can mislocalize partially or completely to the cytoplasm due to genetic and/or environmental factors. Prolonged mislocalization may lead to aggregation and eventually degeneration. Due to the cumulative factors driving toxicity at each stage, reversing TDP-43 pathology may be more challenging or not possible as the neuron approaches degeneration. From (Suk and Rousseaux 2020).

1.4.1 Aggregation

Early studies identified ubiquitin positive inclusions in patients with ALS and FTD that did not co-localize with neither Tau nor alpha-Synuclein, as observed in Alzheimer's Disease and Parkinson's Disease respectively. TDP-43 was identified to be a primary component of such inclusions selective to ALS and FTD(Arai et al., 2006; Neumann et al., 2006). These inclusions are typically characterized by the presence of insoluble TDP-43 and CTFs that can be hyperphosphorylated, particularly at S403/404 and S409/410, and polyUbiquitinated and colocalizing with mRNA, Ubiquitin, and p62(Arai et al., 2006; Buratti and Baralle, 2012; Gal et al., 2007; Mizuno et al., 2006; Nakano et al., 2004; Neumann et al., 2009a, 2006). Much research has focused on the causes and consequences of TDP-43 aggregation and how each of the hallmarks cause toxicity. However, there remains considerable debate around how or whether TDP-43 aggregation drives disease or whether it is a consequence of cellular dysfunction and neurodegeneration(Hergesheimer et al., 2019).

The CTD of TDP-43 plays a key role in its aggregation propensity. A common feature of TDP-43 aggregates is the presence of cleaved forms of TDP-43, specifically C-terminal fragment (CTF) CTF35 and CTF25. Interestingly, these cleaved forms are more commonly found in the brains of ALS/FTD patients than the spinal cord, aligning with TDP-43 pathology observed in disease(Berning and Walker, 2019; Shenoy et al., 2019). Recent structural analyses of TDP-43 aggregates demonstrate that the CTD is crucial for forming an amyloid-like fibrils(Kumar et al., 2023). These amyloid "cores" consist of the TDP-43 CTD, particularly residues 280 to 360 containing a glycine rich region, that forms beta sheet secondary structures and aligning in long polymers forming fibrils. These fibril cores may be essential for seeding activity for prion-like spread of TDP-43 aggregation within and between cells. Studies adding lysate from human brain

samples diagnosed with ALS found that this lysate was sufficient to induce TDP-43 pathology in culture and in mouse models of disease(Nonaka et al., 2013).

A key physiological function of the CTD on TDP-43 is to mediate liquid-liquid phase separation enabling TDP-43 to localize to liquid-like condensates such as nuclear bodies or stress granules(Wang et al., 2020). Mutations within the CTD can alter the liquid properties of TDP-43 condensates, accelerating aggregation(Gopal et al., 2017). A prominent hypothesis to how TDP-43 aggregates form is through phase-transition from liquid to solid TDP-43 granules(Gasset-Rosa et al., 2019; Mann et al., 2019). TDP-43 undergoes LLPS to form liquid TDP-43 granules containing mRNA and are thought to physiologically aid in the coordination of the cellular stress response adjacent to stress granules(Gasset-Rosa et al., 2019; Mann et al., 2019). These TDP-43 granules can readily dissociate upon recovery from cellular stress. However, under prolonged LLPS such as observed in prolonged cellular stress conditions or increased electrostatic interactions through disease causing mutations, TDP-43 will transition into a gel-like inclusion which is not able to readily dissociate(Gasset-Rosa et al., 2019). It is thought that these inclusions may be physiologically removed by the cell, however if not cleared they may transition into a solid state forming a TDP-43 aggregate(Scotter et al., 2014; Wang et al., 2010). Phosphorylation is thought to affect the dynamics of TDP-43 LLPS and may help make TDP-43 more soluble despite initial thoughts that phosphorylation drives aggregation(Haider et al., 2024).

RNA plays a key role in the dynamics of LLPS through electrostatic interactions and generally promotes solubility of aggregation prone proteins including TDP-43(Bevilacqua et al., 2022; Corley et al., 2020; Maharana et al., 2018). Studies have found that TDP-43 RNA binding ability is an important, albeit controversial, mediator of aggregation and toxicity. Loss of RNA binding was found to promotes TDP-43 aggregation suggesting that RNA binding helps maintain

TDP-43 solubility(Chen et al., 2019; Flores et al., 2019; J. C. Necarsulmer et al., 2023). However, some studies argue that RNA binding is a key contributor to aggregation and toxicity as it facilitates LLPS(Ihara et al., 2013; Mann et al., 2019; Rengifo-Gonzalez et al., 2021).

TDP-43 aggregates can also sequester proteins from their native locale. Optogenetic studies using Cryptochrome 2 fusion to TDP-43 to facilitate LLPS found that prolonged LLPS in the cytoplasm eventually causes TDP-43 aggregate formation followed by nuclear depletion and recruitment to aggregates(Asakawa et al., 2020; Gasset-Rosa et al., 2019; Mann et al., 2019; Zhang et al., 2019). Stress granule proteins such as G3BP1 and TIA1, proteins involved in autophagy such as SQSTM1 (p62), and nuclear pore proteins can also be found sequestered in TDP-43 aggregates which result in various deficits within the cell including stress responses, protein clearance, and nucleocytoplasmic transport(Aizawa et al., 2019; Arai et al., 2006; Hirsch-Reinshagen et al., 2017; Keating et al., 2023; Mackenzie et al., 2017; Neumann et al., 2006; Van Deerlin et al., 2008; Yan et al., 2024). TDP-43 aggregates can also directly disrupt cellular processes such as axonal transport which is particularly important in motor neurons and may partially explain their susceptibility in ALS(Alami et al., 2014; Bilsland et al., 2010; Sleight et al., 2020; Svahn et al., 2018).

1.4.2 Mislocalization

Nuclear to cytoplasmic mislocalization is a key aspect of TDP-43 pathogenesis increasingly recognized to occur upstream of aggregation and may serve as a driver of disease. Particularly, mislocalization can result in simultaneous loss of nuclear functions and gain of cytoplasmic function which can promote cellular toxicity and drive disease onset(Suk and Rousseaux, 2020).

Dysregulation of mRNA metabolism, particularly alternative splicing, is a key consequence of TDP-43 loss of function that can affect many cellular processes simultaneously (Buratti, 2008). Indeed, patients living with ALS/FTD with TDP-43 proteinopathy present with significant transcriptomic changes and mis-splicing events (D'Erchia et al., 2017; Krach et al., 2018; Li et al., 2023; Nijssen et al., 2018). More recently it is recognized that TDP-43 plays a critical role in repressing cryptic exon inclusion, a feature increasingly recognized to occur in TDP-43 proteinopathies (Ling et al., 2015). For example, upon TDP-43 loss of function, alternate splicing of *Stathmin 2* (*STMN2*) leads to alternate poly-adenylation and subsequent downregulation of STMN2 protein level. STMN2 is involved in axonal maintenance which is particularly important for motor neurons due to their characteristically long axons (Klim et al., 2019; Melamed et al., 2019). Indeed, loss of *STMN2* is sufficient to drive motor neuropathy in *STMN2* knockout mice, however they do not reproduce the full constellation of ALS-related phenotypes implying other mechanisms are also at play in disease (Baughn et al., 2023; Krus et al., 2022). An additional prominent example of cryptic splicing occurs in *UNC13A*. Loss of TDP-43 function causes cryptic exon inclusion in *UNC13A* altering protein levels (Brown et al., 2022; Ma et al., 2022). Additionally, SNPs in *UNC13A* were long associated with ALS/FTD and were recently found to disrupt TDP-43 binding sensitizing cells to TDP-43 loss of function observed in disease. Recently, biosensors have leveraged TDP-43 cryptic splicing to signal early TDP-43 loss of function (Wilkins et al., 2023; Xie et al., 2024). Studies have also demonstrated that cryptic mis-splicing due to TDP-43 loss of function can result in *de novo* peptide sequences such as in Hepatoma-Derived Growth Factor Related Protein 2 (HDGFL2) (Calliari et al., 2024; Seddighi et al., 2024). Blood serum from ALS patients contain HDGFL2 containing this cryptic peptide.

Antibodies targeted against the unique cryptic peptide enable a functional biomarker of TDP-43 loss of function from blood samples(Calliari et al., 2024).

Several of the DNA specific functions regulated by TDP-43 are also lost upon mislocalization that can contribute to features of ALS/FTD and TDP-43 proteinopathies. As TDP-43 plays roles regulating transcription, loss of nuclear function can cause transcriptomic changes not only through mRNA but through altered transcriptional states(Cox et al., 2010; Schwenk et al., 2016; Takahashi et al., 2013). TDP-43 can also play a key role in regulating transposable elements, particularly prevalent is LINE-1 retrotransposition(Li et al., 2022). Loss of TDP-43 function also plays roles in double stranded break repair by recruiting critical components of the non-homologous end joining complex (NHEJ) to sites of double stranded breaks(Guerrero et al., 2019; Hill et al., 2016; Kim et al., 2020; Riancho et al., 2020). Furthermore, TDP-43 can stabilize R-loops, DNA:RNA hybrids that form during transcription or replication, which protect the genome from damage.(Wood et al., 2020) In tissue and iPSC derived neurons from ALS patients there is evidence of significant accumulation of DNA damage thought to be mediated by TDP-43 loss of function conferred by mislocalization in disease states(Riancho et al., 2020).

While nuclear mislocalization and subsequent nuclear depletion of TDP-43 leads to loss of function, cytoplasmic accumulation can also cause some gain of function effects. However, it is particularly challenging to differentiate cytoplasmic gain of function effects with those of TDP-43 aggregation as they are strongly linked. Upon mislocalization, TDP-43 loss of function leads to altered autoregulation causing an upregulation of *TARDBP* transcripts leading to a homeostatic imbalance(Avendaño-Vázquez et al., 2012; Dykstra et al., 2024; Sugai et al., 2019; White et al., 2018). This can result in the activation of additional clearance pathways, such as TDP-43 cleavage, UPS, or autophagy, or toxic gain of splicing effects if nuclear levels remain too high(Arai et al.,

2010; Fung et al., 2015; Li et al., 2015; Scotter et al., 2014; van Eersel et al., 2011; Wang et al., 2010; Zhang et al., 2009). Increased cytoplasmic and cleaved forms of TDP-43 can accumulate in the mitochondria leading to the release of reactive oxygen species (ROS)(Wang et al., 2019, 2016; Zuo et al., 2021). Additionally, toxic mitochondrial accumulation of TDP-43 releases mtDNA activating the cGAS-STING pathway that can trigger neuroinflammation(Marques et al., 2024; C. H. Yu et al., 2020).

Increased cytoplasmic localization can also play a key role in the cellular stress response, in particular how TDP-43 specifically responds to cellular stressors through LLPS. The increased cytoplasmic accumulation can sensitize how cells respond to cellular stress leading to a hyperactive stress response, potentially due to the excess burden of TDP-43 toxicity or due to reactive physiological roles(Gasset-Rosa et al., 2019; Luan et al., 2023). This can trigger TDP-43 phase separation more readily and can lead to excess TDP-43 accumulation which can phase transition more readily leading to phase transition and aggregation(W. Michael Babinchak et al., 2019).

1.4.3 Potential Mechanisms Initiating Pathogenesis

The initiators of TDP-43 pathology remain unclear. While mutations in *TARDBP* cause ALS/FTD it is not clear how or why the phenocconversion, the conversion from prodrome to clinical symptom onset, typically occurs in elderly individuals(Benatar et al., 2022). Additionally, most cases of ALS/FTD occur independent of *TARDBP* mutations(Goutman et al., 2022). Thus, external factors are likely at play that cause a phenotransition, the transition from phenotypically healthy to prodromal, from normal TDP-43 states to pathological states(Benatar et al., 2022; D'Amico et al., 2013; Niccoli et al., 2017; Yu and Pamphlett, 2017). TDP-43 mislocalization is increasingly recognized to occur upstream of aggregation, thus mechanisms inducing

mislocalization may be key instigators of disease. Aging studies have found that TDP-43 mislocalization occurs with age, particularly in the spinal cord(Hideyama et al., 2012). This may cause sensitivity to other genetic or environmental insults initiating a cascade leading to disease. A key component to this mislocalization is through disruption of nucleocytoplasmic transport by disruption of the nuclear pores(Coyne et al., 2021). Nuclear pore may be disrupted through genetic insults such as loss of Nucleoporin 50 (NUP50), or via dipeptide repeats expressed as a result of *C9ORF72* mutation(Aizawa et al., 2019; Boeynaems et al., 2016; Chou et al., 2018; Freibaum et al., 2015; Jovičić et al., 2015; Majounie et al., 2012; Renton et al., 2011; Zhang et al., 2015). As TDP-43 is a stress-responsive protein, lifelong accumulation of cellular stressors may initiate pathogenesis as cells become less capable at dealing with stress with age. Aging is highly associated with dysfunction of key stress response pathways such as the UPS, DNA damage, and autophagy pathways(Hou et al., 2019). Additionally, increased stress can accumulate with age due to increase ROS production, DNA damage, and unfolded proteins, in addition to external insults such as infections and trauma(Maragkakis et al., 2023). Indeed, excitotoxicity which is highly associated with motor neurons is thought to be a key contributor to motor neuron vulnerability(Odierna et al., 2024; Van Den Bosch et al., 2006). These accumulated insults may push TDP-43 into a prolonged stressed state thereby causing mislocalization and aggregation which can be further compounded by additional insults. Finally, recent evidence suggests that nuclear dysfunction may precede mislocalization. Using RNA aptamers to identify TDP-43 pathology demonstrated that early aggregation may initiate within the nucleus and coincide with nuclear loss of function, specifically mis-splicing of *STMN2*(Spence et al., 2024). Thus, understanding the nuclear mechanisms regulating physiological TDP-43 that may be disrupted early in disease may be key to identify early risk factors and triggers of TDP-43 pathogenesis.

1.5 SUMOylation

SUMOylation is a highly conserved and essential post translational modification involving the covalent binding of Small Ubiquitin-like Modifiers (SUMOs) to lysine residues (Celen and Sahin, 2020; Ilic et al., 2022). SUMO proteins are a part of the larger ubiquitin-like protein family notably including NEDD8 (NEDDylation), ATG8 and ATG12, UMF1 (UMFylation), and ISG15 (ISGylation) which all share nearly identical structure with ubiquitin (Mizushima et al., 1998; Villarroya-Beltri et al., 2017; Zhang et al., 2024; Zhou et al., 2024).

In humans, there are 5 SUMO paralogs, SUMO1 – SUMO5 (Bouchard et al., 2021; Citro and Chiocca, 2013). Mice on the other hand only have 3 paralogs, Sumo1 – Sumo3, which are completely conserved between mice and humans and are ubiquitously expressed in all cell types and tissue (Citro and Chiocca, 2013). SUMO4 and SUMO5 on the other hand display tissue specific expression and are not well characterized (Citro and Chiocca, 2013; Liang et al., 2016). SUMO1 shares ~50% homology with SUMO2 and SUMO3 (Bohren et al., 2004). Mature SUMO2 and SUMO3 share almost 100% amino acid homology, hence they are often referred to as SUMO2/3 as they are very challenging to differentiate without genetic manipulation. Proteins modified by SUMO2/3 can also undergo polySUMOylation resulting in long polySUMO chains (Jansen and Vertegaal, 2021). As SUMO1 does not contain internal SUMOylation sites (i.e. lysine residues), SUMO1 can only be added to cap the polySUMO chain.

The process of SUMOylation mirrors that of Ubiquitination in that it involves an E1, E2, and sometimes E3 ligase cascade (Celen and Sahin, 2020; Ilic et al., 2022). SUMO proteins are first matured via Sentrin Specific Proteases (SENP) through cleavage at the C-terminus to expose a di-glycine motif (Xu and Au, 2005). SENP1 and SENP2 help to mature all paralogs of SUMO whereas SENP4 and SENP5 specifically mature SUMO2/3 (Nayak and Müller, 2014). Next, in an

ATP-dependent manner SUMO is bound to the E1 ligase complex consisting of the heterodimer containing SUMO Activating Enzyme 1 (SAE1) and Ubiquitin-like Activating Enzyme 2 (UBA2) (Gong et al., 1999). SUMO proteins are then transferred to the sole essential E2 ligase, Ubiquitin-like Enzyme 2I (UBE2I) which can facilitate direct SUMOylation of target substrates independently, or with the assistance of E3 SUMO ligases (Desterro et al., 1997; Gong et al., 1997; Johnson and Blobel, 1997; Lee et al., 1998; Saitoh et al., 1998; Sampson et al., 2001; Seufert et al., 1995). Specific E3 SUMO ligases such as ZNF451 show preference to promote polySUMOylation and has been referred to as an “E4 elongase” (Cappadocia et al., 2015; Karvonen et al., 2008). Finally, SUMOylated substrates can be readily deSUMOylated via SENP proteases. In a similar fashion to SENP maturation of SUMO, SENP1 and SENP2 is capable of deSUMOylating all forms of SUMO whereas SENP4 and SENP5 are specific to SUMO2/3 (Nayak and Müller, 2014). Additionally, SENP6 and SENP7 play specific roles in deSUMOylating polySUMO chains.

While SUMOylation can act independently upon substrates, SUMOylation can also interact with a variety of other post translational modifications such as ubiquitination, acetylation, and phosphorylation. PolySUMO chains can be co-modified by ubiquitin molecules, however the consequences of such remain elusive (Jansen and Vertegaal, 2021; Shire et al., 2016). Additionally, polySUMOylation can target a protein for ubiquitination mediated by SUMO-targeted ubiquitin ligases (STUbLs) such as RNF4, RNF111, and TOPORS (Han et al., 2023; Kumar et al., 2017; Liu et al., 2024). Acetylation also occurs on lysine residues, thus there may be competition between SUMOylation and acetylation resulting in functional switches mediated by these modifications (Ullmann et al., 2012). Finally, phosphorylation has been characterized to help facilitate SUMOylation of some proteins, particularly transcription factors (Hietakangas et al.,

2006). These proteins often contain phosphorylation-dependent SUMOylation motifs that require phosphorylation to alter the conformation of the motif to enable SUMOylation. Phosphorylation is also demonstrated to occur on SUMO itself as a modified modifier, however the consequence of such modification is not well characterized(Matic et al., 2008).

1.5.1 Molecular Functions

Broadly, SUMOylation generally functions by facilitating novel interactions by modifying the structures of proteins to form new surfaces for protein-protein interactions, or vice versa to block interactions(Hendriks and Vertegaal, 2016; Lascorz et al., 2022). SUMO proteins are also highly soluble and can help maintain protein solubility in cells, a feature often exploited for protein purification assays *in vitro*(Guerrero et al., 2015; Kuo et al., 2014; Malakhov et al., 2004). SUMO proteins can also function independently as “free” or unconjugated SUMO, interacting with proteins through SUMO interacting motifs (SIMs)(Lascorz et al., 2022). PolySUMOylation can also facilitate protein-protein interactions but can play roles in maintaining solubility in condensates formed by LLPS(Cheng, 2023).

When assessing independent paralogs, SUMO1 is best characterized to be involved in nucleocytoplasmic transport, facilitating substrate interactions with importins and the nuclear pore complexes typically mediated through interactions with RANGAP1 and RANBP2(Evdokimov et al., 2008; Pichler et al., 2002; Pichler and Melchior, 2002). In contrast, SUMO2/3 is best characterized to play roles in response to external or internal stimuli such as cellular stressors, viral response, or DNA damage(Enserink, 2015; Ilic et al., 2022; Niskanen et al., 2015). While the process of SUMOylation is essential in eukaryotes, only knockout of SUMO2, not SUMO1 nor SUMO3, in mammals causes embryonic lethality due to developmental failure of the central

nervous system suggesting paralog specific roles conferred by SUMO2(Wang et al., 2014; Zhang et al., 2008). Additional cellular roles for SUMOylation include regulation of nucleic acid binding, apoptosis, and chromatin accessibility with many new functions being constantly uncovered(Celen and Sahin, 2020).

1.5.2 Roles in the Central Nervous System

Much of what is known about SUMOylation is derived from highly targeted studies analyzing a single substrate of SUMOylation. Additionally, on the cellular and physiological levels, SUMOylation has best been characterized with respect to the cancer field covering functions such as cell division and DNA damage(Celen and Sahin, 2020; Geiss-Friedlander and Melchior, 2007). Much less however is known about SUMOylation with regards to its functional roles in the CNS, despite being essential for CNS development(Wang et al., 2014; Zhang et al., 2008).

SUMO is predominantly localized to the nucleus; however, there has been considerable debate about the role of SUMO beyond the nucleus, particularly at neuronal synapses (Daniel et al., 2018, 2017; Tirard et al., 2012). Indeed, SUMOylation has been characterized to play roles in synaptic functions such as vesicle release, signal transduction, and ion channel function (Colnaghi et al., 2019; Henley et al., 2018; Lee et al., 2014; Marcelli et al., 2017; Matsuzaki et al., 2015; Schorova and Martin, 2016). Many synaptic proteins are characterized to be SUMOylated such as G-coupled protein receptors (GPCRs), Glutamine Receptors (mGluR), Acetylcholine Receptors (AChR), Transient Receptor Potential Villinoid 1 (TRPV1, also known as Capsaicin Receptor), and Dopamine Transporter (DAT)(Henley et al., 2021). Additionally, UBC9 can be found localized to synapses suggesting that active SUMOylation occurs within synapses(Loriol et al., 2014). However, which SUMO paralog(s) are actively SUMOylated at synapses has been debated(Daniel

et al., 2017; Tirard and Brose, 2016). These debates argue that most antibodies demonstrate poor specificity for SUMO paralogs, thus overexpression systems or *in vitro* systems are required to characterize SUMOylated proteins which lead to artifacts(Alexander J. Garvin et al., 2022). Thus, direct evidence *in vivo* is particularly challenging to convincingly demonstrate. Using a knock-in mouse model whereby His₆-HA was knocked into the endogenous *Sumo1* allele, SUMO1 was shown to not localize to synapses *in vivo*, suggesting SUMOylation at synapses are likely mediated by SUMO2 or SUMO3(Tirard et al., 2012; Tirard and Brose, 2016). Critics argued however that the knock-in tag may alter Sumo1 function *in vivo* and does not account for Sumo1 playing indirect roles at synapses(Daniel et al., 2018).

More broadly throughout the brain, SUMOylation has been best characterized with regards to its roles in neurodevelopment(García-Gutiérrez and García-Domínguez, 2022; Ripamonti et al., 2020). A particularly important role for SUMOylation is in regulating neurogenesis and cell differentiation(Queiroz et al., 2024). Additionally, SUMOylation has been implicated in learning and memory as it regulates proteins involved in long-term potentiation such as Nitric Oxide Synthase (NOS) and the immediate early gene *ARC*, in addition to many ion channels and neurotransmitter receptors(Carmichael and Henley, 2018; Du et al., 2020; Henley et al., 2021). *In vivo*, SUMOylation was demonstrated to become activated in the hippocampus in mice that underwent the fear conditioning behaviour test, suggesting that SUMOylation may play roles in memory formation from noxious stimuli(Gustin et al., 2022). During mouse development, SUMO1 levels are elevated then decline as mice age and reach adulthood(Tirard et al., 2012). However, the roles of SUMOylation in late-stage aging remain understudied.

1.5.3 Links to Aging and Neurodegeneration

Understanding the role(s) of SUMOylation in the aging and neurodegeneration has been largely limited to substrate specific studies *in vitro* and in non-mammalian *in vivo* models, likely due to the ability to easily manipulate and study SUMOylation. Broadly, SUMOylation is characterized to regulate cellular processes that are highly associated with aging such as proteostasis, cellular senescence, DNA damage, and autophagy(López-Otín et al., 2013; Princz et al., 2020; Princz and Tavernarakis, 2017; Vijayakumaran and Pountney, 2018). In *C. elegans* models of aging, SUMOylation was demonstrated to play roles in maintaining neuronal longevity and supporting efficient stress responses which typically decline with age(Princz et al., 2020).

Many of the major proteins involved in age-related neurodegenerative diseases are targets of SUMOylation. In Huntington's Diseases, the protein Huntingtin (HTT) is targeted by both SUMO1 and SUMO2 which promotes HTT aggregation and toxicity(Ochaba et al., 2016; O'Rourke et al., 2013; Steffan et al., 2004). Expression of the SUMO Targeted Ubiquitin Ligase RNF4 helped to clear accumulated HTT inferring SUMOylation may have a toxic role in disease(Ohkuni et al., 2018). In relation to Alzheimer's Disease and Parkinson's Disease, Tau and alpha-Synuclein, respectively, are targets of SUMOylation(Dorval and Fraser, 2006). The consequences of SUMOylation with regards to toxicity and the primary SUMO paralogs targeting alpha-Synuclein and Tau yield conflicting results. Similarly to HTT, SUMOylation by SUMO1 is thought to promote aggregation of Tau and alpha-Synuclein(Abeywardana and Pratt, 2015; Dorval and Fraser, 2006; Luo et al., 2014; Vijayakumaran et al., 2015). Several studies focused on SUMO1 have found evidence of SUMO1 colocalization with alpha-Synuclein and Tau aggregates respectively and have concluded that through various processes SUMOylation via SUMO1 promotes aggregation(Abeywardana and Pratt, 2015). The consequences of SUMOylation by

SUMO2, however, yield conflicting results. Knockdown of E3 SUMO ligase Trim28 can reduce toxicity and aggregation of alpha-Synuclein and Tau by inhibiting SUMO2-mediated SUMOylation(Rousseaux et al., 2018, 2016). However recent studies focusing on promoting SUMOylation of Tau with SUMO2 demonstrate neuroprotective effects in Tauopathy mouse models and in response to SARS-CoV-2 infection(F et al., 2022; Orsini et al., 2024). Ultimately, these results emphasize that a more specific understanding of SUMO paralogs and site-specific SUMOylation is required. Other notable proteins involved in neurodegenerative diseases targeted by SUMOylation include Amyloid precursor protein (APP) in relation to Alzheimer's Disease, and DJ-1 in relation to Parkinson's Disease(Shinbo et al., 2005; Zhang and Sarge, 2008).

The relationship between SUMOylation and ALS remains understudied with some associations formed with key proteins in disease, however, there remains little mechanistic insight. SOD1 was found to be SUMOylated by SUMO1 and SUMO3 promoting its aggregation in a similar fashion to HTT, Tau, and alpha-Synuclein (Fei et al., 2006; Niikura et al., 2014). FUS may function as a SUMO E3 ligases, promoting SUMOylation of substrates, its relationship to disease however remains unclear(Oh et al., 2010). Global SUMOylation was shown to be disrupted by DPRs produced by *C9ORF72* HRE(Marmor-Kollet et al., 2020). This disruption of the SUMOylation machinery led to impaired recovery from cellular stress, particularly affecting stress granule disassembly. Thus, SUMOylation may be protective to effectively respond and recover from cellular stress, a critical pathway impaired in ALS/FTD.

1.6 Statement of Research Problem, Rationale, and Objectives

ALS/FTD is a highly complex and heterogenous disease spectrum(Hardiman et al., 2017). While there is a strong genetic component to ALS/FTD, most cases occur sporadically through unknown mechanism. Despite this, TDP-43 pathology is a central feature to nearly all cases of

ALS and almost half of the cases of FTD(Mackenzie and Rademakers, 2008). Many genetic factors that cause ALS/FTD converge on TDP-43 causing dysfunction and contributing to TDP-43 pathology(Hergesheimer et al., 2019; Jo et al., 2020; Liao et al., 2022; Suk and Rousseaux, 2020; Yang et al., 2020). However, it remains unclear why these genetic cases take decades to phenocopy. Additionally, in the absence of clear genetic factors, TDP-43 pathology remains a hallmark of these diseases implying extrinsic factors that accumulate over an individual's lifetime may contribute to converging cellular pathogenic mechanisms.

To maintain longevity, neurons must effectively respond and efficiently recover from cellular stressors(Maragkakis et al., 2023). Disruption of the cellular stress response is highly implicated with ALS/FTD, caused by genetic mutations in disease related genes or excessive stress accumulation, for example through excitotoxicity(Odierna et al., 2024; Van Den Bosch et al., 2006). While TDP-43 is well characterized to play roles in response to cellular stress, the mechanisms regulating TDP-43 under stress remain elusive(Dewey et al., 2011; Lee et al., 2021; Luan et al., 2023; Mark Fang et al., 2019; Walker et al., 2013; Wang et al., 2020; Zuo et al., 2021). SUMOylation is a key post translational modification that helps to facilitate stress responses(Wilkinson and Henley, 2010). Due to the high degree of overlap between functional roles of TDP-43 and those regulated via SUMOylation, *we hypothesized that TDP-43 is a likely target of SUMOylation in response to cellular stress.*

Understanding the global roles of SUMOylation in the brain beyond studies targeted at specific substrates, has been limited by the lack of effective tools to study SUMOylation in the mammalian brain. Typically, studying protein SUMOylation relies on overexpression systems and antibodies with poor target specificity and/or sensitivity(Alexander J Garvin et al., 2022). Tools that facilitate the unbiased study of native SUMOylation in the mammalian brain will help to

uncover specific substrates that help maintain neuronal longevity. *We hypothesized that the generation of a His₆-HA-Sumo2 knock-in mouse model that complements a previously established His₆-HA-Sumo1 model will facilitate the direct comparison of SUMO paralogs in vivo to better understand how each paralog contributes to neuronal function.*

Overall Objective: Uncover implications for SUMOylation in the mammalian brain in health and neurodegeneration.

Objective 1: Determine the functional interplay between TDP-43 SUMOylation, stress response, and ALS/FTD pathogenesis.

Objective 2: Generate and characterize a mouse model to facilitate the study of paralog-specific SUMOylation in the mammalian brain.

Chapter 2: A stress responsive TDP-43 SUMOylation program preserves neuronal function

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2.1 Statement of Author Contributions

T.R.S. and M.W.C.R. conceived study and designed experiments. **T.R.S.** designed, conducted experiments, and analyzed data for most biochemical experiments involving western blots and immunoprecipitations including SUMO assays, primary neuron analyses, and TDP-43^{K408R} mouse characterization. **T.R.S.** and T.T.N. designed, optimized, and performed E3 SUMO ligase screen. **T.R.S.** and B.N. performed and maintained primary cortical neuron cultures. T.T.N. optimized and performed all assays involving Proximity Ligation Assays including in primary neuron culture and human patient tissue. **T.R.S.** and M.W.C.R., through the Center of Phenogenomics at the University of Toronto, designed and generated the TDP-43^{K408R} mouse model. **T.R.S.** maintained mouse colony and generated cohorts for current study with assistance from M.M.H., A.C-G, and S.M.C. **T.R.S.** with assistance from J.L.Z performed and analyzed immunofluorescence assays in primary cortical neuron cultures. C.E.P. planned and performed the battery of behavioural tests and monitored mice during aging study. **T.R.S.** and C.E.P. performed mouse dissections for biochemical and histological analyses. C.E.P. with the assistance of V.S.G. performed and analyzed histological analyses of TDP-43^{K408R} mice. J.M.W., and P.M.M. and J.R. collected and banked human patient samples. **T.R.S.**, C.E.P., T.T.N., J.L.Z., and V.S.G. performed confocal microscopy for immunofluorescent experiments. C.E.P. and V.S.G. performed brightfield microscopy for histological analyses. **T.R.S.** and A.C-G. performed biochemical analyses of human brain samples across aging. **T.R.S.** and M.W.C.R. wrote the manuscript for the study and all authors were involved in editing versions of the manuscripts.

A stress-dependent TDP-43 SUMOylation program preserves neuronal function

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2.2 Abstract

Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) are overwhelmingly linked to TDP-43 dysfunction. Mutations in TDP-43 are rare, indicating that the progressive accumulation of exogenous factors – such as cellular stressors – converge on TDP-43 to play a key role in disease pathogenesis. Post translational modifications such as SUMOylation play essential roles in response to such exogenous stressors. We therefore set out to understand how SUMOylation may regulate TDP-43 in health and disease. We find that TDP-43 is regulated dynamically via SUMOylation in response to cellular stressors. When this process is blocked *in vivo*, we note age-dependent TDP-43 pathology and sex-specific behavioural deficits linking TDP-43 SUMOylation with aging and disease. We further find that SUMOylation is correlated with human aging and disease states. Collectively, this work presents TDP-43 SUMOylation as an early physiological response to cellular stress, disruption of which may confer a risk for TDP-43 proteinopathy.

2.3 Introduction

Altered proteostasis is one of the key hallmarks of aging and is particularly prevalent in neurodegenerative diseases (Hipp et al., 2019). Despite the highly heterogeneous nature of neurodegenerative diseases – likely owing to the extreme genetic and environmental diversity acting upon individuals – a select few proteins are recurrently implicated in pathology (Spire-Jones et al., 2017). TDP-43 is an essential RNA binding protein notorious for its involvement in neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD). In a diseased state, TDP-43 is found mislocalized from the nucleus and aggregated in the cytoplasm of neurons within the central nervous system (CNS) in ~97% and ~45% of ALS and FTD cases, respectively (Grossman et al., 2023; Hardiman et al., 2017). Additionally, ALS and FTD exist on a clinical and genetic spectrum linked by TDP-43 dysfunction often referred together as ALS/FTD (Burrell et al., 2016). Beyond ALS/FTD, TDP-43 pathology is increasingly linked to other neurological disorders including Limbic-predominant Age-related TDP-43 Encephalopathy (LATE), Alzheimer's Disease (AD), Chronic Traumatic Encephalopathy (CTE), and Stroke (Meneses et al., 2021; Nelson et al., 2019; Nicks et al., 2023; Thammisetty et al., 2018). Thus, TDP-43 pathology has garnered much attention to better understand the causes of these diseases and to uncover potential routes of therapeutic intervention.

The accumulation of cytoplasmic TDP-43 aggregates is considered a late-stage event in neurodegeneration. Increasing evidence suggests that the partial mislocalization or complete depletion of TDP-43 from the nucleus to the cytoplasm is an early event in ALS/FTD pathogenesis, functioning independently – however tightly associated – with aggregation (Brown et al., 2022; Coyne et al., 2021; Gasset-Rosa et al., 2019; Klim et al., 2019; Ma et al., 2022; Melamed et al., 2019; Suk and Rousseaux, 2020). As a result, recent efforts have focused on the outcomes of

TDP-43 mislocalization as early markers of dysfunction and/or mechanisms driving disease. Indeed, loss of function due to mislocalization of TDP-43 causes aberrant cryptic splicing in genes including *STMN2* and *UNC13A* that drive the progression of ALS (Baughn et al., 2023; Brown et al., 2022; Klim et al., 2019; Ma et al., 2022; Melamed et al., 2019). To wit, cryptic mis-splicing of *STMN2* was recently found to better correlate with disease progression compared to classical TDP-43 cytoplasmic aggregates (Agra Almeida Quadros et al., 2024; Spence et al., 2024). Additionally, once in the cytoplasm, TDP-43 can exert additional toxicity through gain of function effects by sequestering critical proteins into cytoplasmic aggregates thus disrupting crucial pathways leading to cellular demise (Hergesheimer et al., 2019; Suk and Rousseaux, 2020). Together, dysregulation of TDP-43 is sufficient to drive neuronal dysfunction ultimately leading to neurodegeneration. However, the mechanisms instigating TDP-43 pathogenesis remain convoluted.

Despite the overwhelming prevalence of TDP-43 pathology in ALS/FTD and related diseases, mutations in the gene encoding TDP-43 (*TARDBP*) are only present in less than 1% of all ALS and FTD cases (Zou et al., 2017). It is becoming evident that not one, but many genetic and/or environmental factors affect pathways converging on TDP-43 in ALS/FTD. Many ALS/FTD causative genes exert toxicity by disruption of key pathways such as nucleocytoplasmic transport and cellular stress responses (Ghasemi and Brown, 2018). The cellular stress response is a critical pathway tightly linked to ALS/FTD and TDP-43 supported via genetic, experimental, and epidemiological evidence (Colombrita et al., 2009; D'Amico et al., 2013; Iguchi et al., 2012; Lin and Beal, 2006; Liu-Yesucevitz et al., 2010; Luan et al., 2023; Mackenzie et al., 2017; McDonald et al., 2011; Ratti et al., 2020; Tam et al., 2019; Ueda et al., 2024; Walker et al., 2013; Wolozin and Ivanov, 2019; Zuo et al., 2021). On the one hand, ALS/FTD-linked mutations disrupting various

steps of the stress response pathways converge on the dysregulation of TDP-43 resulting in pathology(Colombrita et al., 2009; Iguchi et al., 2012; Luan et al., 2023; Mackenzie et al., 2017; Tam et al., 2019; Ueda et al., 2024; Walker et al., 2013; Zuo et al., 2021). On the other hand, exogenous insults such as aging, head injuries, viral infections, and other exposures may confer susceptibility or precipitate ALS/FTD and can serve as pre-clinical models of TDP-43 proteinopathy(Ash et al., 2017; Dubinski et al., 2023; Fung et al., 2015; Nicks et al., 2023). Motor neurons, the primary vulnerable cell type in ALS, are thought to be particularly susceptible to stress due to the high levels of excitotoxicity experienced throughout one's lifetime(Odierna et al., 2024). Indeed, prolonged cellular stress due to chronic stress exposure or failures in stress recovery can result in TDP-43 pathology(Ratti et al., 2020; Zhang et al., 2019). However, much less is known about the molecular pathways acting upon TDP-43 in the cell stress response and how they might be linked to age-related neurodegeneration. Uncovering these mechanisms will help to better understand how environmental insults that occur throughout aging converge on TDP-43 and cause susceptibility to disease.

Post translational modifications (PTMs) play key roles in regulating protein function and have been tightly linked to TDP-43 (dys)function and disease. TDP-43 is modified by an array of PTMs including phosphorylation, ubiquitination, acetylation, and polyADP-ribosylation(Arai et al., 2006; McGurk et al., 2018; J. Necarsulmer et al., 2023; Neumann et al., 2009a, 2006). Abnormal TDP-43 phosphorylation and ubiquitination are pathognomonic of ALS and TDP-43 related FTD(Neumann et al., 2009a). Recent studies have suggested that SUMOylation – the covalent conjugation of a Small Ubiquitin-like Modifier (SUMO) to target lysine residues(Henley et al., 2014; Wilkinson and Henley, 2010) – by SUMO1 may have a role in regulating TDP-43 nucleocytoplasmic transport and RNA binding(Maraschi et al., 2021). SUMO1 is best

characterized for its roles in nucleocytoplasmic shuttling(Pichler and Melchior, 2002). SUMO2 however is the only essential SUMO paralog and selectively plays roles in orchestrating cellular stress responses(Enserink, 2015; Wang et al., 2014). Despite the strong relationship between TDP-43 and the cellular stress response, a role for SUMO2 in directly regulating TDP-43 remains elusive.

Here, we show that TDP-43 is modified by SUMO2 selectively in response to cellular stressors. TDP-43 becomes SUMOylated within the nucleus early in response to stress, upstream of TDP-43 aggregation. Modification by SUMO2 is further correlated with dosage and duration of cellular stress and peaks during the recovery phase before it is cleared through the ubiquitin proteasome system. We further identified four E3 SUMO ligases that can modulate the levels of TDP-43 SUMOylation. We found that TDP-43 is SUMOylated by SUMO2 in a conserved region of the C-terminal domain at lysine (K) 408 directly adjacent to phosphorylation residues serine (S) 409/410 characteristically phosphorylated in TDP-43 aggregates. To understand the physiological consequences of blocking TDP-43 SUMOylation, we generated a knock-in mouse model bearing a p.K408R point mutation in endogenous mouse *Tardbp* allele. Cortical neurons cultured from these mice display impaired stress recovery and accumulation of nuclear TDP-43. These mice do not show abnormalities in development but develop social and cognitive deficits as they age. Pathologically, we observe TDP-43 mislocalization and accumulation of phosphorylated TDP-43 in the spinal cord and significant denervation of neuromuscular junctions in aged mice. As SUMOylation of TDP-43 plays a protective role in mice during aging, we assessed human brain samples and observed a positive correlation between global SUMOylation and age inferring an increased demand on SUMO-related pathways during aging. Finally, we observed significant increase in TDP-43 and SUMO2 interactions in the prefrontal cortex from individuals diagnosed

with ALS/FTD compared to unaffected controls suggesting SUMOylation is actively engaged in regulating TDP-43 in disease states.

2.4 Results

TDP-43 is SUMOylated in the nucleus in a context specific manner

Various acute stressors including oxidative (sodium arsenite, NaAsO₂), hyperosmotic (D-sorbitol and sodium chloride), and heat shock have been demonstrated to induce TDP-43 mislocalization and aggregation *in vitro*, helping to uncover key mechanisms affected in disease (Chang et al., 2013; Dewey et al., 2011; Walker et al., 2013). As sodium arsenite is a commonly used stressor to interrogate TDP-43 alterations, we examined whether TDP-43 became SUMOylated in response to sodium arsenite treatment. Using an immunoprecipitation assay (“SUMOylation Assay”, Fig. 1a) to immunoprecipitate HA-SUMO bound proteins under denaturing conditions to disrupt non-covalent protein interactions, we found that TDP-43 becomes SUMOylated by SUMO2 and can form polySUMO chains specifically in response to sodium arsenite (Fig. 1b). Interestingly we found that sodium arsenite and heat shock stressors, but not hyperosmotic stress, induced TDP-43 SUMOylation suggesting that SUMOylation is a context specific modifier of TDP-43 (Fig. S1a).

Previous reports have suggested that TDP-43 is SUMOylated under native conditions by SUMO1 to regulate nucleocytoplasmic transport and RNA binding (Maraschi et al., 2021). Strikingly, we found that TDP-43 SUMOylation was selectively modified by SUMO2 and SUMO3, but not SUMO1, specifically under stressed conditions consistent with the conserved roles of SUMO2 and SUMO3 in response to stress (Fig. S1b). Mature (covalently bound) SUMO2 and SUMO3 share nearly 100% amino acid homology making them difficult to differentiate and thus referred together as SUMO2/3. Since SUMO2 is the only essential and most abundantly expressed SUMO paralog in the CNS and sodium arsenite is commonly used to study TDP-43

pathobiology, we focused on TDP-43 SUMOylation by SUMO2 in response to sodium arsenite stress(Suk et al., 2023; Wang et al., 2014).

TDP-43 and SUMO2 are widely expressed throughout the nucleus however interactions only occur in response to stress, making co-localization analysis challenging to infer SUMOylation events (Fig. S1c). To visualize the interaction between TDP-43 and SUMO2/3 we performed a proximity ligation assay (PLA) in murine primary cortical neurons cultures (Fig. 1c, Fig. S1d). We found that in response to sodium arsenite there is a significant increase in PLA signal between endogenous TDP-43 and SUMO2/3, consistent with the stress-dependent SUMOylation previously observed. To test whether TDP-43 SUMOylation is linked to disease-like states, we expressed ALS-causing mutant TDP-43 (TDP-43^{Q331K}) and disrupted the nuclear localization sequence in TDP-43 (TDP-43^{NLSmut}) to mislocalize TDP-43 to the cytoplasm and found that in response to sodium arsenite TDP-43^{Q331K} leads to increased TDP-43 SUMOylation, while TDP-43^{NLSmut} blocks SUMOylation indicating that nuclear localization is essential for TDP-43 SUMOylation (Fig. 1d). The latter finding is complemented by the nuclear PLA signal further validating that SUMOylation of TDP-43 occurs within the nucleus of cells (Fig. 1c). Taken together, SUMOylation may be an important mechanism regulating nuclear TDP-43 upstream of mislocalization in response to stress.

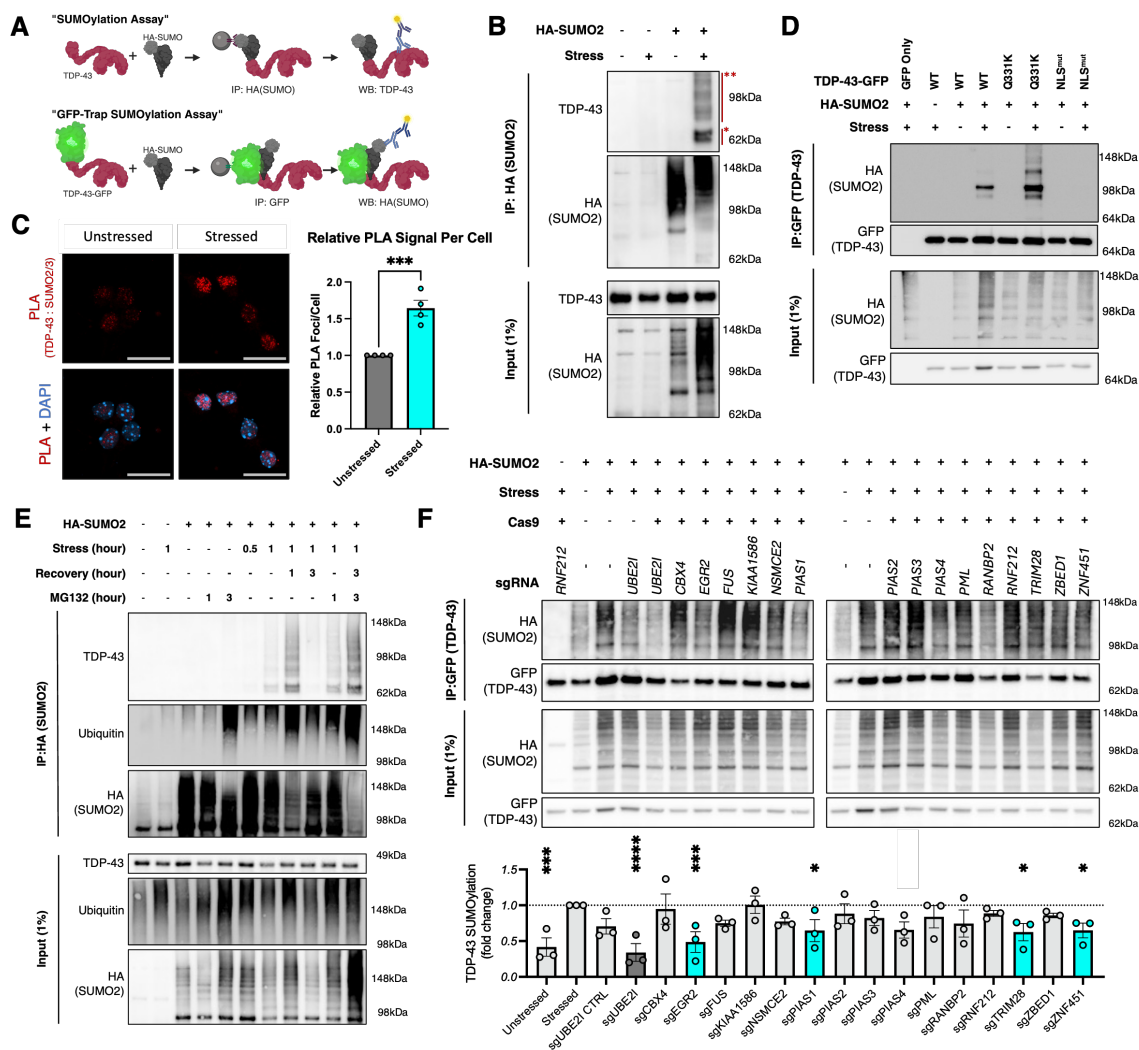


Fig. 1: SUMOylation dynamically regulates nuclear TDP-43 in a stress-responsive manner.

(A) Schematic of immunoprecipitation assays to detect TDP-43 SUMOylation. **(B)** Representative SUMOylation Assay western blot detecting TDP-43 SUMOylation specifically in response to 1 hour sodium arsenite (250 μ M) stress. * = SUMOylated TDP-43, ** = PolySUMOylated TDP-43. **(C)** Representative image and quantification of relative proximity ligation signal between TDP-43 and SUMO2/3 in murine primary cortical neuron cultures in response to 1 hour sodium arsenite (250 μ M) treatment. Scalebar = 20 μ m. (N = 4 per condition) Unpaired T-test data presented as mean $\pm$ SEM, *** p<0.0005 **(D)** Representative GFP-Trap SUMOylation assay depicting

increased SUMOylation with ALS-causing Q331K mutation and blocked SUMOylation by disruption of the Nuclear Localization Sequence (NLS^{mut}) in response to 250 μ M sodium arsenite stress. **(E)** Representative SUMOylation Assay of TDP-43 SUMOylation dynamics during stress (250 μ M sodium arsenite) and recovery demonstrating a dependency on the ubiquitin proteasome for clearance of polySUMOylated TDP-43 by treatment with proteasome inhibitor MG132 (2 μ M). **(F)** Representative GFP-Trap SUMOylation assay screening for potential E3 ligases regulating TDP-43 SUMOylation in response to 1 hour sodium arsenite stress (250 μ M). (N = 3) RM One-Way ANOVA with Fisher's LSD test data presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

SUMOylation is an early event in response to stress and helps clear TDP-43 through the ubiquitin proteasome system during recovery

Prolonged cellular stress *in vitro* leads to mislocalization of TDP-43 and progressive transition into insoluble aggregates resembling aspects of pathology observed in disease (Gasset-Rosa et al., 2019; Iguchi et al., 2012; Mann et al., 2019; Ratti et al., 2020; Zuo et al., 2021). As TDP-43 SUMOylation occurs within the nucleus upstream of nuclear egress, we postulated that SUMOylation would occur upstream of aggregation during prolonged sodium arsenite stress. To test this hypothesis, we performed a time course assay to characterize the dynamics of TDP-43 SUMOylation during prolonged treatment with sodium arsenite. We observed that TDP-43 becomes readily SUMOylated in the first 15-30 minutes of the acute stress response upstream of the accumulation of RIPA-insoluble, phosphorylated TDP-43 (Fig. S1e). Furthermore, we observed an increase in TDP-43 SUMOylation that correlated with the duration of stress. To determine whether SUMOylation occurs in response to relative stress intensity, we performed a dose response assay and found that TDP-43 SUMOylation is proportional to relative stress intensity (Fig. S1f). Therefore, the increase in TDP-43 SUMOylation during prolonged stress is likely due to an increased stress burden over time.

To determine the fate of SUMOylated TDP-43, we performed a stress-recovery time-course assay where cells were stressed with sodium arsenite for up to 1 hour, then allowed cells to recover after washout. For the first hour during stress recovery, we observed a peak in TDP-43 SUMOylation indicating that TDP-43 continues to be SUMOylated and polySUMOylated during stress recovery (Fig. 1e). SUMOylated TDP-43 was not detectable after 3 hours of recovery, suggesting that most of the clearance occurs between hours 2-3 post stress. TDP-43 is known to interact with the SUMO-Targeted Ubiquitin Ligase RNF4 which can polyUbiquitinate

SUMOylated proteins to be degraded through the UPS (Keiten-Schmitz et al., 2020). To determine whether the UPS functions to clear SUMOylated TDP-43 during stress recovery, we treated cells with the proteasome inhibitor MG132 and monitored TDP-43 SUMOylation during stress and recovery. We observed that SUMOylated TDP-43 was not cleared during post-stress recovery with the addition of MG132 indicating that SUMOylated TDP-43 is marked for degradation by the UPS pathway. Importantly, treatment with MG132 for 1 or 4 hours was not sufficient to induce TDP-43 SUMOylation by itself, consistent with the stress-selective nature inducing TDP-43 SUMOylation. Taken together, TDP-43 SUMOylation occurs early in the stress response which then leads to ubiquitin-targeted clearance during stress recovery demonstrating SUMOylation may be an important mechanism regulating nuclear TDP-43 proteostasis.

Stress-dependent TDP-43 SUMOylation is mediated by select E3 SUMO ligases

E3 SUMO ligases are important mediators for SUMOylation of select substrates and enable spatiotemporal regulation of this modification. Thus, they may represent critical modulators of TDP-43 SUMOylation. To identify potential E3 SUMO ligases that may regulate TDP-43 SUMOylation, we first performed a literature search to identify potential ligases with evidence of mediating SUMOylation. We selected 15 candidate E3 SUMO ligases and using a dual sgRNA/Cas9 approach generated knockout cell lines for each of the candidate ligases and performed GFP-Trap SUMOylation assays to test the effects on TDP-43 SUMOylation (Fig. 1f) (Chu and Yang, 2010; Eisenhardt et al., 2015; García-Gutiérrez et al., 2011; Kagey et al., 2003; Kahyo et al., 2001; Karvonen et al., 2008; Kotaja et al., 2002; Liang et al., 2011; Nakagawa and Yokosawa, 2002; Oh et al., 2009; Payne et al., 2014; Pichler et al., 2002; Reynolds et al., 2013; Sachdev et al., 2001; Yamashita et al., 2016). As a positive control, we knocked out the sole E2

SUMO ligase, UBC9 (encoded by *UBE2I*), which led to a near complete loss of TDP-43 SUMOylation demonstrating the functionality of the approach. We identified four SUMO E3 ligases whose knockout consistently reduced the levels of stress-induced TDP-43 SUMOylation: *EGR2* (*KROX20*), *PIAS1*, *TRIM28* (*KAP1*), and *ZNF451* (*ZATT*). *EGR2* is linked to the neuromuscular disorder Charcot Marie Tooth Disease and is an immediate early gene rapidly reacting to external cellular stimuli aligning with the early response of TDP-43 SUMOylation upon stress (García-Gutiérrez et al., 2011; Warner et al., 1998). *PIAS1* is a canonical, highly conserved E3 SUMO ligase known to play roles in cellular stress responses (Nishida and Yasuda, 2002). *TRIM28* is also a well characterized E3 SUMO ligase which is a predicted interactor of SUMOylated TDP-43 based on the GPS-SUMO2.0 algorithm (Liang et al., 2011; Ren et al., 2009; Zhao et al., 2014). *ZNF451* is characterized as a stress dependent E3 SUMO ligase specifically promoting polySUMOylation with SUMO2/3. It is sometimes referred to as an “E4 SUMO elongase” which complements our data that TDP-43 is polySUMOylated in response to stress (Eisenhardt et al., 2015; Karvonen et al., 2008). Identification of these putative TDP-43 SUMO ligases supports the robustness of stress-induced TDP-43 SUMOylation and adds layers of nuance into the regulation of TDP-43 with variable routes of modulating TDP-43 functions.

TDP-43 SUMOylation occurs in a conserved region of the C-terminal domain

SUMOylation occurs at lysine (K) residues often residing within a consensus SUMOylation motif, Ψ -K-x-D/E (Ψ =large hydrophobic residue, x = any amino acid), however it is increasingly recognized that SUMOylation at lysine residues in non-consensus motifs is not uncommon and plays significant roles in regulating protein function (Zhao et al., 2014). Using GPS-SUMO (<https://sumo.biocuckoo.cn/>) to predict likely SUMOylation sites on TDP-43, we identified a

consensus SUMOylation motif within the first RNA recognition motif at K136 – thought to be the site targeted by SUMO1 – and a non-consensus SUMOylation motif within the C-terminal domain at K408(Ren et al., 2009; Zhao et al., 2014). We performed a GFP-Trap SUMOylation assay and expressed mutant forms of TDP-43-GFP blocking the predicted sites by mutating the lysine residue to similarly charged arginine residues: K136R, K408R, and double mutant 2KR. We observed that TDP-43^{K136R} did not block sodium arsenite-induced TDP-43 SUMOylation, however the TDP-43^{K408R} mutant significantly reduced TDP-43 SUMOylation, suggesting K408 is the primary site of SUMOylation in response to sodium arsenite stress (Fig. 2a). Importantly, mining two independent unbiased mass spectrometry studies identifying sites of SUMOylation across the proteome also revealed TDP-43 SUMOylation at K408, supporting our findings (Hendriks et al., 2018; Lumpkin et al., 2017).

To address the evolutionary conservation of TDP-43 SUMOylation, we aligned the amino acid sequences of TDP-43 with 300 orthologs from OrthoDB and found that human K408 is nearly completely conserved throughout jawed-vertebrates concurrent with the evolution of the C-terminal domain (Fig. 2b, Supplementary Table 1). While this domain harbors the majority of ALS/FTD causing mutations, the residues surrounding K408 display considerably high levels of conservation (~86% pairwise identity) indicating that this motif may play important roles in regulating TDP-43 in vertebrates that may be altered by disease causing mutations (Fig. 2c, Supplementary Table 1). Consistent with these findings, no missense variants have been identified that disrupt the SUMOylation motif (based on gnomAD v4.0(Chen et al., 2023)) supporting its robust conservation in the healthy human population.

The residues surrounding TDP-43 K408, specifically S403, S404, S409, and S410 are characteristically phosphorylated in patients with TDP-43 proteinopathy(Neumann et al., 2009a).

To determine whether phosphorylation of TDP-43 at these serine residues interact with SUMOylation at K408, we expressed phospho-dead “4SA” TDP-43 (S403/404/409/410A) and observed that TDP-43^{4SA} exhibits increased SUMOylation following stress (Fig. 2d). Whether phosphorylation functions allosterically to remove SUMO2/3 at K408 or antagonistically to block the SUMOylation recognition site remains unclear. The independent modification by either phosphorylation or SUMOylation may serve as a mechanism to differentially regulate TDP-43 function independently or sequentially in response to stimuli. Thus, while TDP-43 phosphorylation has gained significant attention since its initial discovery (Neumann et al., 2009a) – in part due to the availability of phospho-specific antibodies – we now show that the C-terminal domain contains a conserved “PTM-enriched region” where SUMOylation at K408 precedes phosphorylation, revealing additional complexity and nuance to the stimulus-induced regulation of TDP-43.

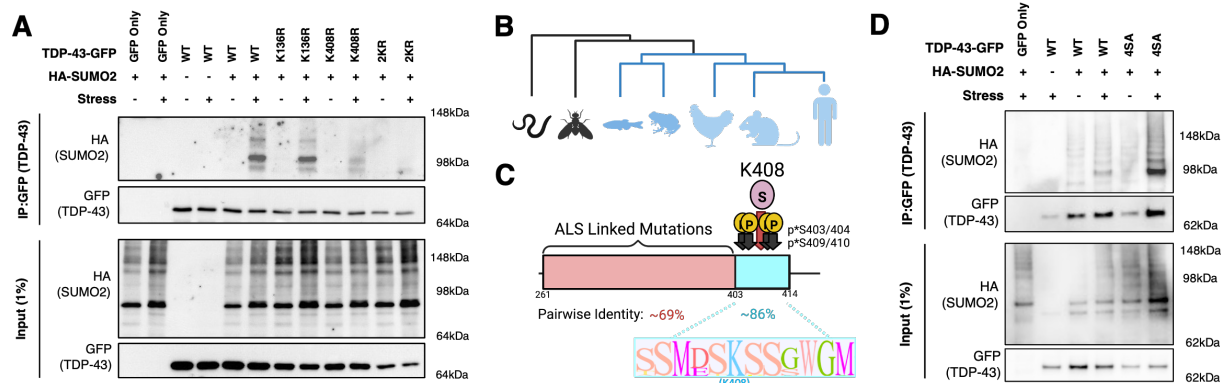


Fig. 2: TDP-43 is SUMOylated at K408 in a conserved region of the C-terminal domain.

(A) Representative GFP-Trap SUMOylation assay to map the site of TDP-43 SUMOylation in response to 1 hour sodium arsenite (250 μ M) stress. **(B)** Phylogenetic representation from alignment of representative TDP-43 paralogs highlighting the emergence and conservation of [human] K408 (Blue). **(C)** Pairwise identity of the [human] TDP-43 C-terminal domain from MUSCLE alignment of 300 amino acid sequences for TDP-43 paralogs from Humans to Actinopterygii from OrthoDB. Red represents the TDP-43 C-terminal domain (Intrinsically disordered domain). Cyan represents PTM- enriched region at the extreme C-terminus. **(D)** Representative GFP-Trap SUMOylation assay with phospho-dead TDP-43 “4SA” (S403/404/409/410A) highlighting antagonism between SUMOylation and phosphorylation.

TDP-43 SUMOylation at K408 supports cellular stress response and recovery

To understand the cellular and physiological roles of TDP-43 SUMOylation, we generated a knock-in mouse model harboring a missense (c.1223 A>G) point mutation in the endogenous mouse *Tardbp* locus resulting in the expression of Tdp-43 bearing a p.K408R missense mutation thereby blocking endogenous Tdp-43 SUMOylation (Fig. 3a, Fig. S2a,b). For simplicity herein, all genes/proteins will be referred to following their human nomenclature (e.g. mouse Tdp-43 versus human TDP-43, collectively “TDP-43”). Although TDP-43 and SUMO2 are independently essential for embryonic development, the “TDP-43^{K408R}” mice are born at normal mendelian ratios without gross impairment indicating that TDP-43 SUMOylation at K408 is not essential for murine development (Fig. S2c)(Kraemer et al., 2010; Wang et al., 2014).

As TDP-43 SUMOylation responds to, and is cleared during cellular stress and recovery respectively, we posited that SUMOylated TDP-43 plays key roles in responding and recovering from stress. We cultured murine TDP-43^{+/+} and TDP-43^{K408R/K408R} primary cortical neurons and performed stress and recovery assays to determine how the cell stress response may be affected. Since TDP-43 is critical for the formation of G3BP1 positive stress granules, we examined their formation and resolution following stress and recovery(Khalfallah et al., 2018; Sidibé et al., 2021). We found that stress granules could form in TDP-43^{K408R/K408R} neurons to the same degree as TDP-43^{+/+} neurons, but there was a significant delay in stress granule clearance during recovery (Fig. 3b, Fig. S3a-c). When assessing TDP-43 solubility, we also observed a significant increase in RIPA insoluble phosphorylated TDP-43 at 1 hour recovery that was cleared by 3 hours post-stress recovery (Fig. 3c). Based on our previous results that SUMOylation is cleared by the UPS pathway and phosphorylation may function antagonistically with SUMOylation, the increase in phosphorylation during recovery may serve as an alternative method of proteostasis to compensate

for loss of SUMOylation. We further observed a significant increase in nuclear TDP-43 foci that were specific to the recovery phase and were exacerbated after 3 hours recovery in the TDP-43^{K408R/K408R} neurons (Fig. 3b, Fig. S3d). Various forms of nuclear TDP-43 bodies are increasingly recognized to be aberrantly regulated in disease and stress states (Shelkovnikova et al., 2018; Spence et al., 2024; Wang et al., 2020; Yu et al., 2021). Together, this may indicate that TDP-43^{K408R/K408R} neurons appropriately respond to, but not efficiently resolve cellular stress which can render cells vulnerable to further insult.

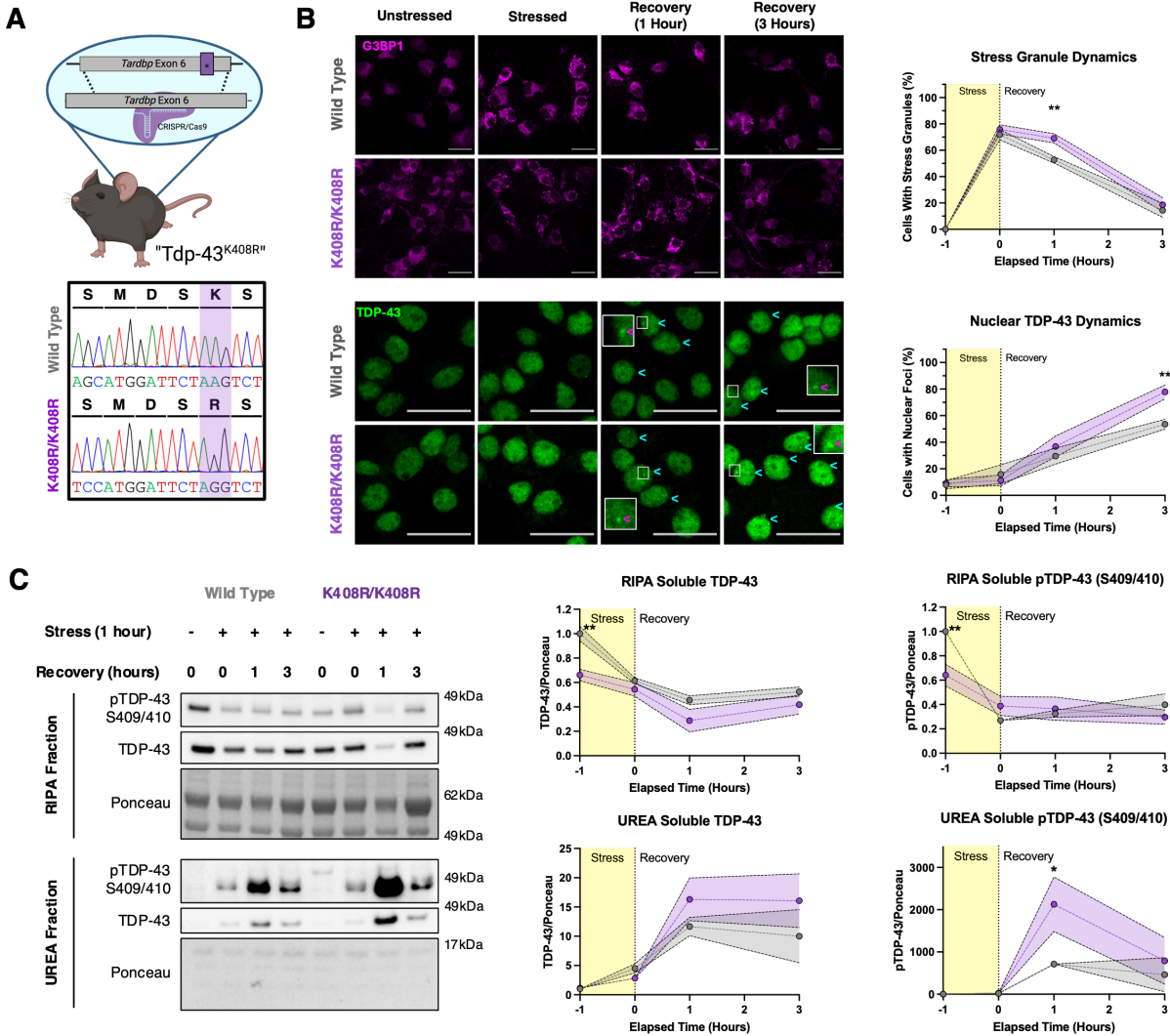


Fig. 3: Blocking TDP-43 SUMOylation at conserved K408 impairs the cellular stress response.

(A) Schematic and sanger sequencing of the TDP-43^{K408R} knock-in mouse line. **(B)** Representative images and quantification of G3BP1 stress granule dynamics and TDP-43 nuclear foci formation in mouse primary cortical neurons (7 DIV) during stress (1 hour 250 μ M sodium arsenite) and recovery. Cyan arrowheads denote cells with nuclear TDP-43 foci, magenta arrow in insets denote TDP-43 foci. G3BP1 contrast was set for optimal visualization of stress granules. Scale Bar = 25 μ m. (N = 5), 2-Way ANOVA with Fisher's LSD test data presented as mean $\pm$ SEM, ** p<0.005.

(C) Representative western blot and quantification of RIPA and UREA fractions from mouse primary cortical neurons (7 DIV) during stress (1 hour 250 μ M sodium arsenite) and recovery. N=3-5, 2-Way ANOVA with Fisher's LSD test data presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.005$.

Blocking TDP-43 SUMOylation in vivo leads to sex-specific social and cognitive deficits

The process of aging is known to progressively induce stress on cells, particularly in the nervous system, as cellular functions become impaired or as environmental triggers accumulate over time (Hipp et al., 2019). As TDP-43 SUMOylation is involved in supporting the normal cellular stress response and maintaining TDP-43 proteostasis, we hypothesized that disrupting this process leads to age-dependent physiological changes accelerated by impaired stress responses and TDP-43 accumulation. To ensure that the K408R mutation did not lead to post gestational developmental changes that would confound the presentation of age-related impairment, we assessed the weights and motor abilities of pups at P21. We did not observe any significant differences between TDP-43^{+/+}, TDP-43^{K408R/+}, or TDP-43^{K408R/K408R} with respect to body weight or motor abilities indicating that blocking TDP-43 SUMOylation does not lead to gross developmental abnormalities (Fig. S4a-c).

To determine whether blocking TDP-43 SUMOylation leads to phenotypes in an age-dependent manner, we performed a battery of behavioural tests focusing on motor, social, and cognitive function related to ALS and FTD at three time points (2, 9 and 16 months, Fig. S4d-f, 5-7, Supplementary Table 2). We observed sex-specific cognitive and social effects biased to female animals that generally worsened with aging. Starting around 2 months of age, we observed significant barbering in female mice leading to alopecia in the K408R mutants (Fig. 4a). Consistent with this finding we found that aged female TDP-43^{K408R/K408R} mice were significantly more submissive to their TDP-43^{+/+} counterparts in the tube test (Fig. 4b). Thus, blocking TDP-43 SUMOylation leads to social abnormalities in female mice.

In assessing the cognitive behaviours, we observed significant risk taking and hyperactivity in the open field test at 2 months of age (Fig. 4c). This hyperactivity was age-dependent and

selectively affected young female mice as they were significantly less active than their TDP-43^{+/+} counterparts at 9 months of age in the open field tests (Fig. 4c). Additionally, these female TDP-43^{K408R/K408R} mice presented with significant impairment during habituation at 9 months in the beam break test (Fig. 4d). As there was no change in motor abilities in the rotarod and digigait tests (Fig. S6c,d), we rationalized that the activity abnormalities were likely due to cognitive and anxiety impairments as opposed to motor impairments. By 16 months of age, female TDP-43^{K408R/K408R} mice performed significantly worse than TDP-43^{+/+} mice in the spontaneous Y-maze, inferring age-dependent cognitive decline in the domain of working memory (Fig. 4e). Together, female mice present with social and cognitive impairment that progresses throughout aging. There were no significant cognitive effects in male TDP-43^{K408R/K408R} mice, however mild impairments were observed on the rotarod test at 9 months of age (2-way ANOVA $p=0.0273$, Supplementary Table 2). This deficiency was not observed at 16 months of age, largely due to all mice performing significantly worse at the rotarod task, limiting the interpretation of this assay. Taken together, the age-dependent cognitive and social impairment observed in female TDP-43 SUMO-deficient mutants highlight interesting and unexplored aspects of TDP-43 with important implications for understanding sex-specific roles of TDP-43 SUMOylation.

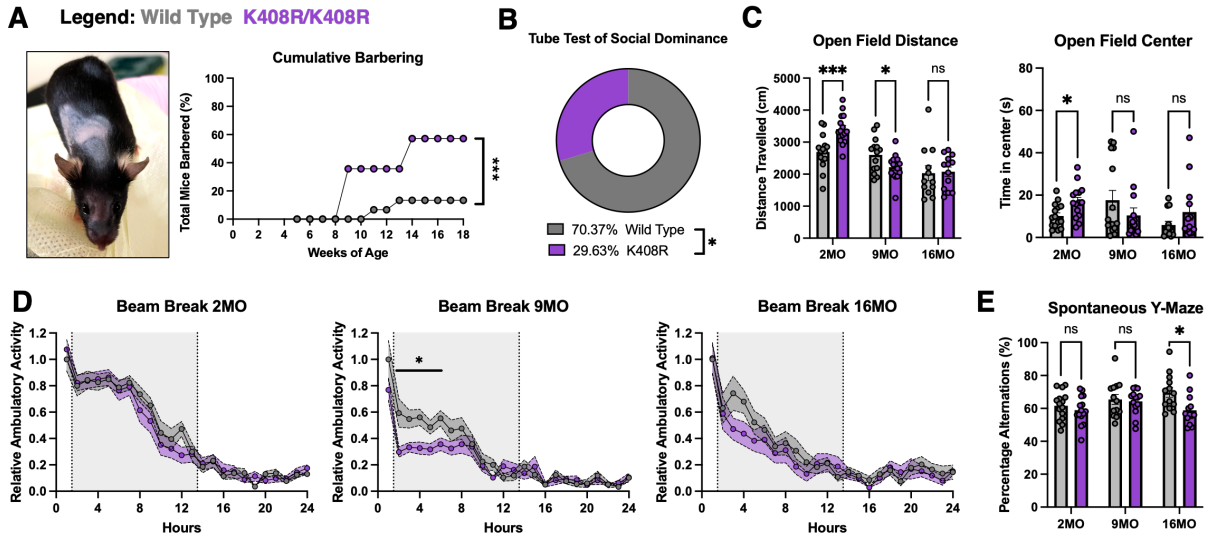


Fig. 4: Blocking TDP-43 SUMOylation *in vivo* leads to sex-specific social and cognitive impairment.

(A) Representative image of a barbered female mouse and quantification of cumulative barbering probability. 2-Way ANOVA with Tukey's Multiple Comparisons, *** $p < 0.0005$. (B) Tube test of social dominance at 16 months of age (16MO) displaying the total head-to-head battles and win percentage by genotype comparing Wild Type against K408R (K408R/+ and K408R/K408R animals). Binomial Test of Observed vs. Expected with Expected due to random chance set to 50%, * $p < 0.05$. (C) Quantification of total distance traveled (cm) and total time in center (s) in the open field test. Mixed Effects Analysis with Tukey's multiple comparison data presented as mean $\pm$ SEM, *** $p < 0.0005$, * $p < 0.05$. (D) Quantification of percent alternations in the spontaneous Y-maze test at 2, 9, and 16 months of age (2MO, 9MO, 16MO, respectively). Mixed-Effects Analysis with Tukey's multiple comparison data presented as mean $\pm$ SEM, * $p < 0.05$. (E) Quantification of relative ambulatory activity normalized to Wild Type at 1 hour at 2, 9, and 16 months of age (2MO, 9MO, and 16MO, respectively). Grey shading between hours 2 and 14

indicate “lights off” with respect to day/night light cycle. 2-Way ANOVA with Tukey’s multiple comparison data presented as mean $\pm$ SEM, * $p < 0.05$.

TDP-43^{K408R} mice present with distinct features of TDP-43 pathology related to ALS and FTD

Our biochemical and cellular assays show that stress-induced SUMOylation helps to clear TDP-43 during recovery and blocking TDP-43 SUMOylation in neurons leads to impaired recovery from stress and TDP-43 accumulation (Fig. 1e, 3). Due to the sex-specific social and cognitive impairment in female mice, we posited that TDP-43 accumulation would reflect the behavioural changes. Consistent with the sex-specific social and cognitive impairment described above, we find that insoluble phosphorylated TDP-43 accumulated in the cortex of female, but not male mice (Fig. S8a,b). Furthermore, this accumulation was observed as early as 2 months of age and was maintained throughout aging, implying that TDP-43 accumulation is initially driving the progressive behavioural changes.

Cellular and molecular changes are generally thought to precede behavioural changes in mouse models and human disease. We questioned whether motor neurons in the spinal cord present with early-stage pathology as they are subject to excess excitotoxic stress throughout their lifespan (Odierna et al., 2024). Surprisingly, we observed cytoplasmic mislocalization, but not nuclear depletion, of TDP-43 accompanied by insoluble, phosphorylated TDP-43 accumulation in the lumbar spinal cord of both male and female mice at 9 months of age (Fig. 5a, Fig. S8c,d). Furthermore, by 16 months of age, male TDP-43^{K408R/K408R} displayed a significant denervation of neuromuscular junctions in the tibialis anterior and a reduction in ChAT positive neurons in the spinal cord compared to littermate controls (Fig. 5b,c, Fig. S8e-g). Together, spinal motor neurons are vulnerable to impaired TDP-43 SUMOylation.

As the TDP-43^{K408R} mice continued to age we observed a 24.49% decrease in survival specific to male TDP-43^{K408R/K408R} mice with a median survival of 521 days for the TDP-43^{K408R/K408R} compared to 690 days for TDP-43^{+/+} mice (Fig. 5d, Fig. S4d). Mice were typically found dead or

euthanized after reaching a humane endpoint. Taken together, our data supports that TDP-43 SUMOylation plays a protective role in mediating TDP-43 proteostasis in the CNS and that its blockade may confer a risk for ALS/FTD-like pathogenesis in an age-dependent, and sex-specific manner providing insights into molecular substrates underlying sexually dimorphic clinical features of ALS and FTD.

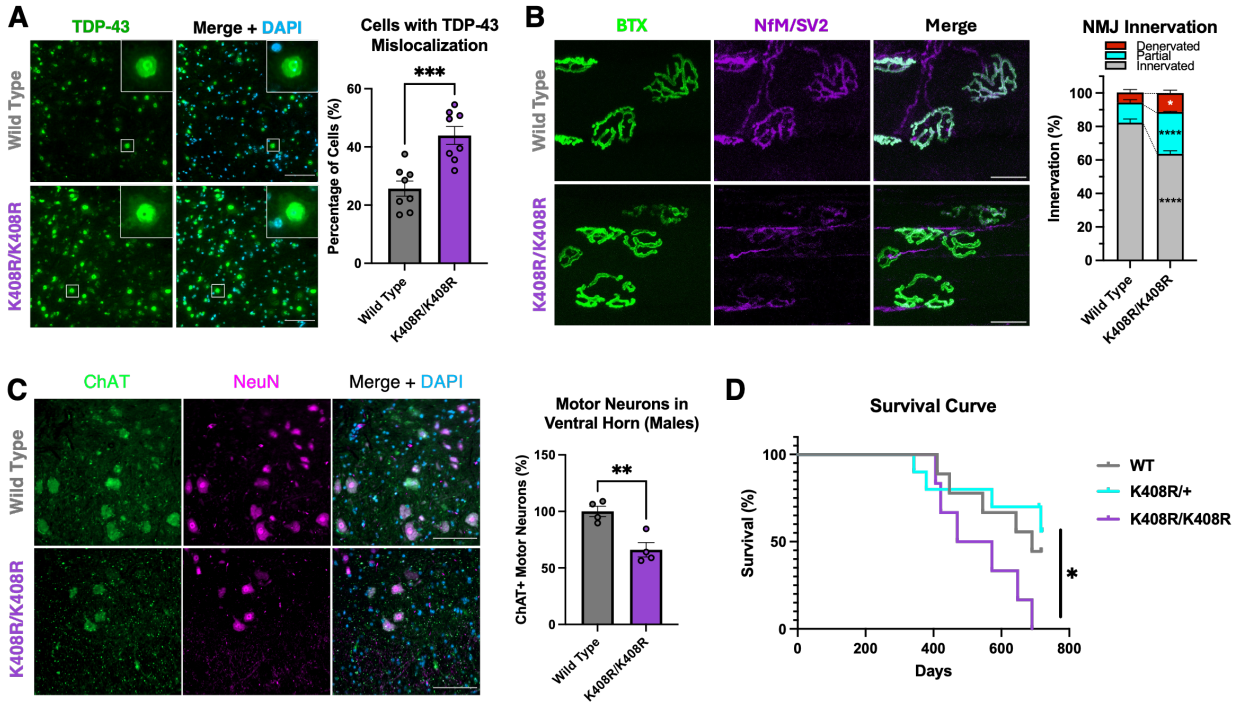


Fig. 5: Male TDP-43^{K408R} mice present with age-specific features of ALS.

(A) Representative images and quantification of TDP-43 mislocalization in the lumbar spinal cord of 9-month-old TDP-43^{K408R} mice. Each datapoint represents the average of 4 serial sections 40 μ m apart per individual mouse. Scalebar = 100 μ m. (N = 4 per sex/genotype) Unpaired t-test, *** p < 0.0005. See Supplementary Fig. 9c for sex comparisons. (B) Representative images and quantification of neuromuscular junction (NMJ) innervation in the tibialis anterior of 16-month-old TDP-43^{K408R} mice. >80 NMJs were quantified per animal (N = 4 per genotype). 2-Way ANOVA with Tukey's multiple comparison analysis, *p < 0.05, ****p < 0.0001. (C) Representative images and quantification of ChAT+ motor neurons in the ventral horn of the lumbar spinal cord of male mice. Each datapoint is the average of 4 serial sections spaced 40 μ m apart through the lumbar enlargement of the lumbar spinal cord. Unpaired t-test, ** p < 0.005. (D) Survival curve for male TDP-43^{K408R} mice (Females found in Supplementary Fig. 4d). Curve comparisons analyzed using Log-Rank test and Gehan-Breslow-Wilcoxon test. *p < 0.05.

Aberrant SUMOylation is a feature of human aging and disease

Although SUMOylation is well understood to play an essential role in the CNS, its involvement in human aging and neurodegenerative diseases remains largely elusive. To assess how SUMOylation is normally affected in human aging, we processed samples from human temporal lobe tissue ranging between 0.5 and 88 years of age from individuals unaffected by neurological diseases (Fig. 6a, Supplementary Table 3, n=55). Complementing our findings that SUMOylation serves as a response to increased proteostatic demands, such as those in our aging TDP-43^{K408R} model, we observed that global SUMOylation was also positively correlated with age (Fig. 6b).

To explore the extent of TDP-43 SUMOylation in human ALS/FTD cases, we performed proximity ligation assays against TDP-43 and SUMO2/3 in prefrontal cortex samples from ALS/FTD cases positive for TDP-43 pathology and age/sex matched unaffected controls (Supplementary Table 3). Surprisingly, we observed a significant increase in TDP-43 – SUMO2/3 interactions in the prefrontal cortex of ALS/FTD patients with TDP-43 pathology (Fig. 6c, Fig. S9a). This may be a result of increased stress burden throughout the prefrontal cortex in those affected by ALS/FTD with TDP-43 pathology compared to age and sex matched counterparts. Taken together, SUMOylation as a stress responsive modification is positively correlated with aging in the human brain, potentially linked to the increase demand imposed by age-associated stress. Additionally, the SUMOylation response is enhanced in the frontal cortex of those affected by ALS/FTD providing further *in vivo* support for its role in disease.

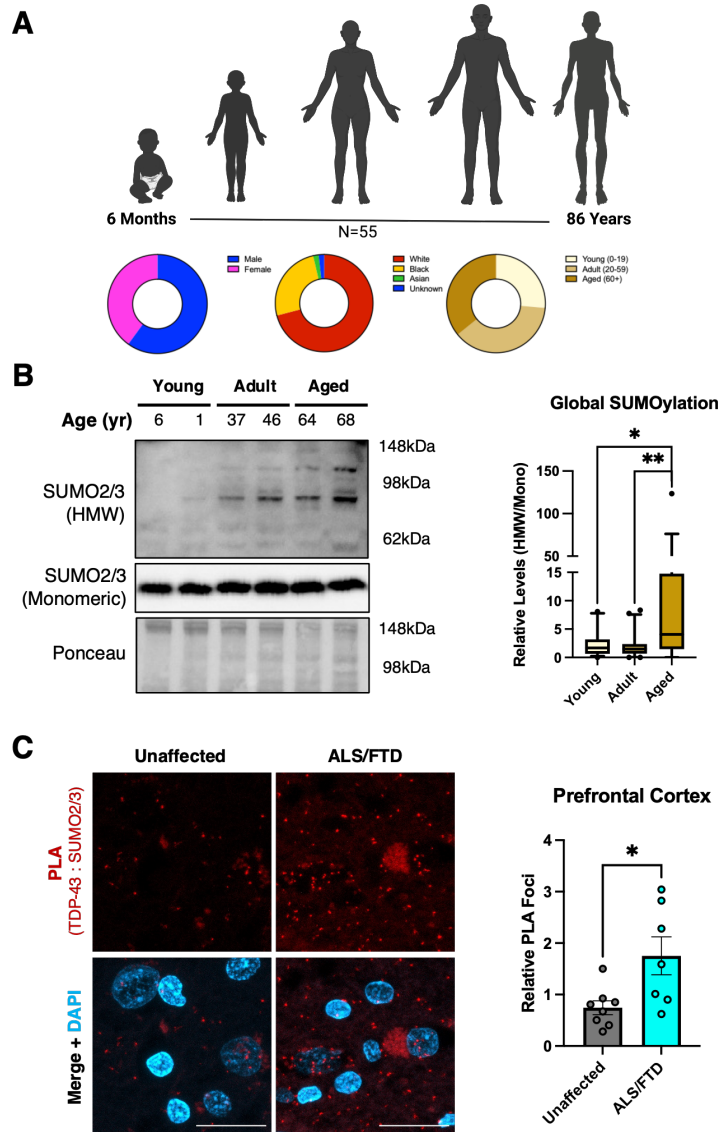


Fig. 6: SUMOylation correlates with aging and is enriched in the prefrontal cortex of ALS/FTD patients.

(A) Demographic composition of temporal lobe samples from aging cohort. (B) Representative western blot and box plot of SUMO2/3 across binned age groups. Average Age: “Young” = 6.32yrs, N = 14; “Adult” = 31.11yrs, N = 20; “Aged” = 64.00yrs, N = 19. Error bars indicate 10-90 percentile. Kruskal-Wallis with Uncorrected Dunn’s multiple comparisons test. * $p < 0.05$, ** $p < 0.005$. (C) Representative images and quantification from proximity ligation assay (PLA)

between SUMO2/3 and TDP-43 in the prefrontal cortex. Unaffected patient ID: NP23-02, Female, 74 yrs. ALS/FTD patient ID: A14-101, Female, 67 yrs. Scale bar = 25 μ m. (N = 7-8) Unpaired T-test data presented as mean $\pm$ SEM, * $p < 0.05$.

2.5 Discussion

Although ALS and FTD are highly heterogeneous diseases, the nearly universal convergence on TDP-43 proteinopathy emphasizes a need to understand early drivers of this pathogenic process. Mutations in various genes can lead to TDP-43 pathology, however the majority of ALS and FTD cases occur sporadically without a clear genetic cause (Ghasemi and Brown, 2018; Hardiman et al., 2017). Additionally, patients living with disease causing mutations do not present with symptoms of disease until later in life suggesting exogenous factors related to aging play a role in phenocopy (Yu and Pamphlett, 2017). Cellular stressors are well characterized to induce TDP-43 pathology providing a clue as to how exogenous factors may contribute to disease pathogenesis. Given the postmitotic nature of neurons, maintaining proteostasis in response to lifelong stressors is critical for neuronal longevity (Hipp et al., 2019). This is particularly true for motor neurons which endure constant excitotoxic stress (Odierna et al., 2024). In this study, we found that SUMOylation maintains TDP-43 proteostasis in the nucleus specifically in response to stress. This modification is critical to preserve neuronal function, the absence of which results in neuronal demise and TDP-43 proteinopathy.

SUMOylation of TDP-43 occurs at K408 in a highly conserved region of the C-terminal domain. This is in striking contrast to what was previously reported for TDP-43 and SUMO1; where it was suggested, but not demonstrated, that SUMO1 modified TDP-43 at lysine 136 (Maraschi et al., 2021; Maurel et al., 2020). Indeed, mutating K136 to an arginine (R) had no effect on stress-induced TDP-43 SUMOylation by SUMO2/3. In genetic forms of ALS or FTD caused by mutations in TDP-43, the C-terminal domain harbors the overwhelming majority of disease-causing mutations (Kirola et al., 2022). Thus, understanding the involvement of the C-terminal domain in TDP-43 biology is critical to uncover pathways that may be linked to disease

independent of TDP-43 mutations. Phosphorylation of TDP-43 in the C-terminal domain, particularly S403/404 and S409/410, have garnered much attention in this regard as these residues are characteristically phosphorylated in disease (Neumann et al., 2009a). However, the roles for phosphorylation at these sites remains generally elusive. Few reports have suggested roles in regulating liquid-liquid phase separation and solubility (Brady et al., 2011; Wang et al., 2018). Our findings suggest that phosphorylation and SUMOylation act antagonistically on TDP-43, indicating potential distinct mechanisms of regulation. We further observed that this region of the C-terminal domain targeted by SUMOylation and phosphorylation is highly conserved throughout vertebrate species. Thus, this PTM-enriched region may play key roles for regulating TDP-43 in vertebrate biology.

Regulation of TDP-43 proteostasis is critical for cells as fluctuations in TDP-43 levels can lead to toxicity in cell and animal models (Campbell et al., 2021; Hergesheimer et al., 2019; Milstead et al., 2023; Wegorzewska et al., 2009; Yang et al., 2022). Unsurprisingly, TDP-43 is regulated in a variety of mechanisms including transcriptionally, post-transcriptionally, translationally and post-translationally (Buratti, 2018; Hergesheimer et al., 2019; Suk and Rousseaux, 2020). Furthermore TDP-43 can be cleared through several pathways including the proteasome, endo-lysosome, autophagy, and extracellular vesicle pathways. Here we add another mechanism of regulation through SUMOylation during stress and recovery. We hypothesized that blocking TDP-43 SUMOylation with K408R mutation would result in its accumulation during stress recovery. Interestingly, we observed a significant increase in insoluble phosphorylated TDP-43 in TDP-43^{K408R/K408R} cortical neurons and a delay in stress granule disassembly during stress recovery indicating TDP-43 accumulation. However, this was resolved 3 hours post-stress indicating that the neurons could compensate to clear the aberrantly accumulated TDP-43 within

this acute stress paradigm. We posit that phosphorylation of TDP-43 may be an additional mechanism to regulate TDP-43 proteostasis in the event of SUMOylation-pathway failure. Thus, SUMOylation may be an early line of defense to regulate TDP-43 in response to stress and blocking TDP-43 SUMOylation shifts proteostasis leading to delayed recovery. Future studies exploring repeated and/or divergent stressors will be crucial in determining the degree to which TDP-43 SUMOylation safeguards from cellular demise.

Previous reports have emphasized the status of altered proteostasis as a hallmark of aging in the CNS, culminating in impaired removal of damaged proteins with age(Hipp et al., 2019). In TDP-43^{K408R/K408R} mice, TDP-43 was found to accumulate in the CNS in an age-dependent manner, suggesting SUMOylation plays important roles during aging. Supporting these findings, in human temporal lobe samples we similarly observed a positive correlation between global SUMOylation and age supporting SUMOylation as a potential protective mechanism during aging in the CNS. Previous studies on global SUMOylation have focused on the dynamic nature of this modification playing roles during development, and associations between SUMOylation and neurodegeneration gaining traction however roles for SUMOylation in the central nervous system during the process of human aging remain understudied(Princz and Tavernarakis, 2017; Yau et al., 2020). This may be ascribed, in part, to challenges in studying SUMOylation due to the dynamic nature of the modification and lack of specific tools to study its interactions. We employed a PLA approach to assess the interaction between TDP-43 and SUMO in ALS/FTD patients and found significantly increased interactions in the prefrontal cortex relative to age/sex matched unaffected controls. Recent studies have used RNA aptamers to reveal nuclear changes in TDP-43 in spinal and cortical neurons of ALS patients prior to traditional cytoplasmic pathology in vulnerable cells(Spence et al., 2024). Further development of these technologies with respect to TDP-43

SUMOylation may help advance biomarker development that can provide information about TDP-43 stress-response and pathogenesis in ALS/FTD patients.

The emergence of predominantly cognitive FTD-like phenotypes in the TDP-43^{K408R} mice is not unusual compared to other TDP-43 knock-in models such as TDP-43^{Q331K} and TDP-43^{K145R} mice which present with features of cognitive dysfunction but lacking the entire constellation of ALS/FTD-like phenotypes (J. Necarsulmer et al., 2023; White et al., 2018). As TDP-43 SUMOylation plays roles in regulating TDP-43 clearance during recovery from stress, blocking TDP-43 SUMOylation in the TDP-43^{K408R/K408R} mouse line disrupts this proteostasis and leads to subsequent mislocalization consistent with loss of nuclear function. Additionally, we observe significant increases in insoluble phosphorylated TDP-43 and motor neuron loss in male mice suggesting that gain of function may be occurring, phenocopying disease pathogenesis. While we did observe some motor impairment in 9-month-old mice correlating with TDP-43 mislocalization, we did not observe significant motor impairment in the male mice at 16 months of age, which may be explained by the overall poor performance during motor tests at this age limiting the sensitivity to detect mild changes. Despite this, we did observe molecular and histological evidence supporting early motor impairment with significant neuromuscular junction denervation and reduction in ChAT positive motor neurons in the ventral horn. Additionally, as the male TDP-43^{K408R/K408R} mice displayed a significant decrease in survival, behavioural analysis at 16 months of age may have preceded the onset of significant motor phenotypes. Future refined studies using key pathological markers centered around key pathological markers of cellular dysfunction will help uncover specific points of phenoconversion and phenotransition to identify the chronology of events leading to phenotypes associated with neurodegeneration.

We initially hypothesized that aging may be a sufficient stressor to interrogate TDP-43 SUMOylation in mice, the various stressors that individuals with ALS/FTD were previously exposed to during their lifetime (i.e. the “exposome”) are not experienced by mice in vivaria on a short timescale. TDP-43^{K408R} model will therefore facilitate the exploration of how the exposome synergizes with TDP-43 to better understand how ALS/FTD relevant stressors (e.g. *C9ORF72* expansions, viral infections, and traumatic brain injury) converge on TDP-43 and uniquely drive aspects of neurodegeneration.

2.6 Materials and Methods

Materials Availability

Plasmids generated in this study will be deposited to Addgene by the time of publication.

Mouse lines generated in this study are available upon request.

Data and Code Availability

Human tissue data reported in this paper will be shared by the lead contact upon request.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Cloning of pEGFP-TDP-43 plasmid and variants

For expression of C-terminally tagged TDP-43-EGFP, human TDP-43 was subcloned out of a wtTDP-43tdTOMATOHA (gift from Zoushang Xu, Addgene # 28205) via XhoI and KpnI double digest and inserted into a pEGFP-N3 (Clontech) backbone and validated by sanger sequencing. Site directed mutagenesis was performed using QuikChange II XL (Agilent 200521) following manufacturer instructions. Primers for site directed mutagenesis can be found in table S4. Several TDP-43 variants were generated through the uOttawa Genome Engineering and Molecular Biology facility or designed and ordered through twist bioscience and were subcloned into the pEGFP-TDP-43 backbone for consistency using XhoI and HindIII double digest, see table S4 for plasmids used/generated in this study.

Cell Culture

HEK293T cells were cultured in DMEM outgrowth media containing 10% FBS (unless otherwise stated) and antibiotic/antimycotics at 37°C with 5% CO₂.

Generation of pL302-HA-SUMO2 stable HEK293T cell line

To generate a pL302-HA-SUMO2 lentivirus-compatible vector for stable HA-SUMO2 expression, HA-SUMO2 was subcloned from pcDNA-HA-SUMO2 (gift from Guy Salvesen, Addgene #48967) and inserted into the pL302 lentiviral backbone (gift from Jacqueline Burré and Thomas Südhof at Stanford). Briefly, extension PCR was performed to add an XbaI restriction site to the 5' and 3' ends of the HA-SUMO2 insert using PCR amplification using forward primer sequence: 5'- ggctgcaggctgactctagaaagcttatggatgcctaccc-3', and reverse primer sequence: 5'- tggctgcaggctgactctagatgcatgctcgagtcgaacct-3'. The PCR amplicon and pL302 backbone were digested with XbaI and the amplicon was ligated into the pL302 backbone. Colonies were screened by sanger sequencing to ensure proper orientation and copy number of the insert. Next the pL302-HA-SUMO2 plasmid was packaged into lentivirus by co-transfection of psPAX2 (Gift from Didier Trono, Addgene #12259), and pMD2-G (Gift from Didier Trono, Addgene #12260) into HEK293T cells and subsequent media collections. Viral media was filtered through a 0.45 mm filter then concentrated by centrifugation at 100,000G for 2 hours at 4 °C and the pellet was resuspended in 1X PBS. Fresh HEK293T cells were transduced with pL302-HA-SUMO2 virus to generate a stable polyclonal cell line.

GFP-trap SUMOylation assay

~250,000 HEK293T and/or pL302-HA-SUMO2 HEK293T cells were seeded (experiment

dependent) in a 6-well plate. The following day, plasmids were transfected to express TDP-43-GFP (or mutant variants) and/or HA-SUMO variants and allowed to incubate for 48 hours for optimal expression. Cells were treated with 250 mM NaAsO₂ for one hour (or variations of time, concentration, or chemical depending on assay), after which cells were immediately collected by scraping the bottom of the well and pelleting the samples by centrifugation at 2500G for 5 minutes at room temperature. The supernatant was then aspirated, and the cells were flash frozen or immediately lysed in 450 uL of ice cold 1X Denaturing Lysis buffer (1X RIPA, supplemented with 1X protease inhibitor, 1X phosphatase inhibitor, 50mM N-Ethylmalamide, and 5% 2-mercaptoethanol) and boiled at 95 °C for 5 minutes. After boiling, 450 uL of ice cold 1X Lysis buffer (1X RIPA, supplemented with 1X protease inhibitor, 1X phosphatase inhibitor, 50mM N-Ethylmalamide) was then added to the sample, and cells were left on ice for 20 minutes with vortexing every 5 minutes for 10 second. Next, the cell lysates are centrifuged at ~21000G for 20 minutes at 4 °C and during this time, 30 uL of GFP-Trap beads (Bulldog Bio) per sample were washed 3 times in cold 1X RIPA buffer then aliquoted equally into microcentrifuge tubes for each sample. Following the centrifugation, 1-3% of the supernatant was transferred into a microcentrifuge tube to be used as the *Input* sample. The remainder of the supernatant was transferred into a microcentrifuge tube containing the GFP-Trap beads as the immunoprecipitation sample and was then placed on a rotator for 45 minutes at 4 °C. After the incubation, the solution was aspirated, and the beads were washed using cold 1X RIPA buffer 5 times. Samples were eluted in 2X Laemmli buffer containing 10% Beta-Mercaptoethanol and boiled at 85 °C for 10 minutes at 1250 rpm on a thermomixer. The input samples were prepared with 4X Laemmli buffer containing 10% Beta-Mercaptoethanol and then boiled at 85 °C for 10 minutes. Samples were then analyzed by Western Blot.

Endogenous SUMOylation assay

~500,000 HEK293T and/or pL302-HA-SUMO2 HEK293T cells were seeded (experiment dependent) in a 6-well plate. The following day, cells were treated respective of their experiment after which cells were immediately collected by scraping the bottom of the well and pelleting the samples by centrifugation at 2500G for 5 minutes at room temperature. The supernatant was then aspirated, and the cells were flash frozen or immediately lysed in 100 uL of 1% SDS buffer (1% SDS in IP Lysis Buffer supplemented with 1X protease inhibitor, 1X phosphatase inhibitor, 100mM N-Ethylmaleimide), vortexed for 20 seconds then boiled at 95°C for 10 minutes. After boiling, 900 uL of ice-cold IP Lysis buffer was then added to the sample, and cells were left on ice for 20 minutes with vortexing every 5 minutes for 10 seconds. Next, the cell lysates are centrifuged at ~21000G for 20 minutes at 4 °C. Following the centrifugation, 1-3% of the supernatant was transferred into a microcentrifuge tube to be used as the *Input* sample. The remainder of the supernatant was transferred into a microcentrifuge tube containing 1 to 10 ug of antibody per sample (See table S5 for concentrations) and incubated overnight rotating at 4 °C. The next day, the solution was added to a magnetic rack kept at 4 °C and the supernatant was collected for *flowthrough* to test immunodepletion or aspirated. The beads were washed 5X in ice cold IP Lysis buffer. Samples were eluted using 5 ug of synthetic HA peptide (Sino Biological) per 1ug of antibody and incubating at 37 °C for 15 minutes at 900rpm on a thermomixer (for anti-HA immunoprecipitations) or by boiling in 2X Laemmli buffer containing 10% Beta-Mercaptoethanol at 85 °C for 10 minutes at 1250rpm on a thermomixer (for other endogenous immunoprecipitations). The input/flowthrough and HA immunoprecipitation samples were prepared with 4X Laemmli buffer containing 10% Beta-Mercaptoethanol and then boiled at 85 °C

for 10 minutes. Samples were then analyzed by Western Blot using light chain specific secondary antibodies.

Serial protein extraction from cell culture

Soluble protein was extracted using RIPA buffer (supplemented with protease inhibitor, phosphatase inhibitor, and 50mM N-Ethylmaleimide) during immunoprecipitation assays (taken as *Input* prior to immunoprecipitation) or from lysate in cortical neuron experiments. The remaining pellet after the centrifugation step post-lysis was washed in 1mL RIPA buffer with vortexing for 10 seconds followed by centrifugation at ~21000G for 20 minutes at 4 °C. The supernatant was carefully removed, and the pellet was resuspended in 300 mL of 2% SDS buffer in PBS for immunoprecipitation assays, or in 300 mL of 8M UREA in PBS with 10mM Tris-HCl pH 7.4. Samples were left to solubilize overnight at room temperature before 2X Laemmli was added prior to Western Blot analysis.

Western blot analysis

Protein sample prepared in Laemmli loading buffer was loaded onto 8% polyacrylamide gel, TGx Mini-PROTEAN 4-15% precast gel (BioRad), or Bolt Bis-Tris Plus mini-Protein Gel 4-12% (Invitrogen) and run at 100-140 constant voltage in Tris-Glycine or MES buffer for their respective gels. Proteins were transferred onto a 0.45 mm nitrocellulose membrane at a constant 340 mA for 2 hours at 4 °C. The membranes were blocked in 10% milk diluted in TBS-T, washed 5 x 5 minutes in TBS-T then incubated in primary antibody overnight (table S5). The following day the membranes were washed for 5 x 5 minutes in TBS-T, then incubated in secondary antibody for 1-2 hours at room temperature. Finally, the secondary antibody is washed 5 x 5 minutes in TBS-T

before being imaged using chemiluminescence Clarity Western ECL or Clarity Max Western ECL on an LAS4000 (GE). Densitometry was performed using the volumes function of the ImageLab (BioRad) software.

E3 SUMO Ligase Screen

One day prior to transfection 62,000 HEK293T or HEK293T stably expressing HA-SUMO2 were plated in a 6 well dish. Samples were co-transfected using 800 ng of pCLIP-Dual-sgRNA for each of the SUMO E3 ligases alongside 1200ng pLenti-Cas9-BLAST and media was changed after 4 hours to minimize toxicity. Forty-eight hours after transfection, cells were selected using 2 mg/mL puromycin for 2-3 days. After selection was complete, cells were recovered in outgrowth media. On day 7 post transfection, 6-well plates were coated with 10 mg/mL poly-D-lysine for 1 hour at 37 °C then washed 3X in 1X PBS. 200,000 cells were plated into a 6 well dish for each knockout condition and respective controls. The following day, 500ng of pEGFP-N3 or pEGFP-TDP-43 was transfected for each respective condition and media was changed after 4 hours. Cells were incubated for 48 hours (10 days post Cas9/sgrNA transfection). Cells were then stressed for 1 hour using 250 mM sodium arsenite and GFP-Trap SUMOylation Assay and Western Blot analysis was performed as previously described. Samples were quantified using densitometry normalizing each condition to the stressed positive control condition on each respective gel.

Conservation analysis of TDP-43 K408

Representative FASTA amino acid sequences of TDP-43 paralogs were aligned for phylogenetic analysis using MUSCLE multiple alignment and PhyML maximum likelihood for phylogenetic tree generation (Dereeper et al., 2008). Pairwise alignment of the C-terminal domain was

performed using Geneious Prime 2022.1.1 (<https://geneious.com>) performing a MUSCLE multiple alignment of 300 FASTA amino acid sequences of TDP-43 paralogs between Humans and Actinopterygii representing the emergence of [human] K408 curated from the OrthoDB database (table S1).

TDP-43^{K408R} mouse line generation

At The Centre of Phenogenomics (SickKids, Toronto, ON, Canada), the *TDP-43^{K408R}* mice were generated on a C57BL6/N background based on previous methods (Gertsenstein and Nutter, 2018) using CRISPR/Cas9-mediated gene editing of the endogenous *Tardbp* locus. In brief, spCas9 loaded with an sgRNA to target exon 6 of *Tardbp* (*MGI:2387629*; 5'-*TGGGGGCTTTGGCTCGAGCA-3'*) was electroporated into embryos alongside a single stranded oligonucleotide (ssODN) repair template (5'-*CTAAATCTACCTAACCTAATAACCAACCTACTAACCACCCCCACCACCTACATTCCCCAGCCAGAAGACcTAGAATCCATGgaCGAGCCAAAGCCCCATTAAAACCACTGCCCCGATCCTGCATTTGATGCTGACCCCCAACCAAGGGGGGC-3'*). Embryos were screened via allelic discrimination (see “*TDP-43^{K408R}* genotyping” below) to identify founders. Four founders were crossed to C57BL6/N mice to ensure germline transmission of knock-in allele.

Mouse husbandry

All mouse procedures were carried out in accordance with the Canadian Council on Animal Care and approved by the University of Ottawa Animal Care Committee. All mice were group housed (3-5 per mice cage) with access to food and water *ad libitum* on a standard 12-hour light dark cycle. Exceptions included adult male mice who were separated and single housed if persistent

fighting and fight wounds were observed in the cage. All experimental mice were given crinkle paper in addition to the standard nestlet and hut enrichment material. Husbandry was completed by University of Ottawa Animal Care and Veterinary Services except for cohorts actively undergoing behaviour testing for which husbandry was completed by the experimenter.

Mouse wellness monitoring

Mice were monitored weekly starting at age P21 and included assessment of hindlimb clasping, kyphosis, weight, and general health. The hindlimb function was assessed following previous methods (Miedel et al., 2017). Briefly, the mouse was suspended in the air for approximately 5-10 seconds by the base of the tail. Features of the limbs were assessed to give a score from 0-4. Kyphosis was assessed by allowing the mice to briefly walk on the flat table top in the housing room and visually observing the straightness of the spine. A score of 0-3 was given based on previously described methods (Guyenet et al., 2010). Weight was measured every other week by placing individual mice on a digital weigh scale. Notably, mice exhibiting barbered patches were monitored and recorded.

Sex and age of experimental mice

Experiments were performed using both male and female mice sufficiently powered to detect sex differences. In some instances where no sex differences were observed, male and female mice were grouped together to assess genotypic differences independent of sex. Experiments involving primary cortical neurons cultures dissected from individual embryos were used involving a mixture of male and female embryos. Experimental animals underwent behavioural testing as early as P21 and as late as 16 months of age. See Table S2 for number of animals used (distinguished

by sex and genotype), raw behaviour data, and specific statistical analyses for each behaviour experiment.

TDP-43^{K408R} genotyping

Tail samples were collected prior to weaning and again postmortem for genomic DNA (gDNA) isolation and genotyping. Tails were solubilized in 300 µL solubilization buffer (10X SET, 100 mM NaCl, 100 µg/mL Proteinase K (Bio Basic PB0451-250)) at 55 °C overnight. Cell debris was precipitated by adding 150 µL of “Tail Salts Buffer” (4.31M NaCl, 0.63M HCl, 10 mM Tris-HCl, pH7.4) then samples were centrifuged at ~21,000g for 15 minutes at 4 °C. Supernatant containing gDNA was transferred into 600 µL of chilled 100% ethanol to precipitate nucleic acids which were then pelleted by centrifugation for 10 minutes at ~21,000g at 4 °C. The supernatant was carefully removed, and the pellet was washed in 600 µL of chilled 70% ethanol then centrifuged at ~21,000g at 4 °C for 5 minutes. The supernatant was carefully removed, and residual supernatant was left to evaporate for 5 minutes. The gDNA pellet was resuspended in double distilled H₂O at 55 °C for 10 minutes. Genotyping reaction was prepared in a 10 µL reaction containing ~5ng of gDNA, 250nM *K408R Genotyping Forward* primer (5'-CCACCATTCTAAATCTACCTAACCTAATA-3'), 250nM *K408R Genotyping Reverse* primer (5'-GGATCGGGCAGTGGTTTTTA-3'), 125nM Wild Type Locked Nucleic Acid (LNA) probe (*HEX-TCT+A+A+GT+CT+TCT+GGC-IowaBlack FQ*), 125nM K408R LNA probe (*FAM-TCT+A+G+GT+CT+T+CT-IowaBlack FQ*), and 2X PerfeCTa qPCR Tough Mix (QuantaBio). Reactions were run on a BioRad CFX96 qPCR thermocycler with the following cycling parameters: Initial annealing at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds then 60 °C for 60 seconds. Results were analyzed via allelic discrimination in the BioRad Maestro software. See Fig. S2b.

Primary cortical neuron cultures

Pregnant mice were euthanized between gestation E13.5–15.5 with 120 mg/kg Pentobarbital Sodium (Bimeda-MTC, 8015E) delivered via intraperitoneal injection. The embryos were removed and placed into ice cold 1X PBS. For each embryo, the cortices were carefully isolated and meninges removed and then placed in ice cold HBSS (Sigma Aldrich). Cortices were dissociated for 20 minutes with trypsin (Thermo Scientific) at room temperature on a rotator before trypsin inhibitor with DNase solution was added to quench the reaction. Cells were pelleted at 2,500 x g for 5 minutes at 4°C. The supernatant was carefully removed and the pellet was washed with trypsin inhibitor plus DNase solution. Cortical neurons were pelleted at 2,500 x g for 5 minutes at 4°C. The supernatant was carefully removed and the neurons were resuspended in 1 mL of ice cold outgrowth media (Neurobasal media (Thermo Scientific), supplemented with 1X B-27 (Thermo Scientific), 1X N-2 (Thermo Scientific), 500 µM L-Glutamine (Wisent Bioproducts), and 0.5% penicillin/streptomycin (GE Healthcare Life Sciences)). Culture dishes and coverslips were prepared in advanced coated with 50 µg/mL Poly-D-Lysine overnight at 37 °C specific to each experiment detailed below. Cultures were maintained for 7-9 days *in vitro prior (DIV) to experimentation*.

Murine Primary Neuron Culture Maintenance

Primary cortical neurons were dissected and maintained in Neurobasal media (Thermo Scientific), supplemented with 1X B-27 (Thermo Scientific), 1X N-2 (Thermo Scientific), 500 µM L-Glutamine (Wisent Bioproducts), and 0.5% penicillin/streptomycin (GE Healthcare Life Sciences) at 37°C with 5% CO₂. Each neuron culture was harvested from a single embryo representing one

biological replicate. The sex of the embryos/cultures were not determined thus experiments contain a mix of both male and female replicates.

Immunofluorescence in primary cortical neurons

Micro Coverglass #1.5 coverslips (Electron Microscopy Sciences) coverslips were washed in 2M HCl overnight at 55 °C, washed 5 times in sterile H₂O, then pre-coated with poly-D-lysine (50 µg/mL) overnight at 37 °C, then washed with distilled water three times and air-dried at room temperature for at least 2 hours. Primary mouse cortical neurons were seeded at 75,000-100,000 cells per coverslip and cultured as described above. On day 7, neurons were fixed for 10 minutes using 10% phosphate buffered formalin for (Fisher Chemical, SF100-4) followed by 3 x 5-minutes washes in 1 mL of 1X PBS. Neurons were blocked in 500 µL of blocking buffer (1% Triton X-100, 10% cosmic calf serum in 1X PBS) for 1 hour, then incubated in 300 µL of primary antibody diluted in blocking buffer overnight at 4 °C. The following day, the neurons were washed for 5 x 5-minutes in 1 mL of 1X PBS then incubated in 300 µL of secondary antibody diluted in blocking buffer for 2 hours at room temperature. Neurons were then washed in 1 mL 1X PBS, then stained with DAPI for 10 minutes at room temperature followed by 4 x 5-minutes washes in 1X PBS before being mounted using antifade fluorescence mounting media (Dako, S3023).

Proximity ligation assay in primary cortical neurons

Primary mouse cortical neurons were cultured and fixed as described in *primary cortical neuron cultures* and *immunofluorescence in primary cortical neurons*. Fixed coverslips were transferred into 12-well plates and outlined with a hydrophobic pen and blocked using 40 µL of Duolink blocking buffer (Sigma Aldrich, DUO82007) at 37 °C for 1 hour. Coverslips were then washed for

3 × 5 minutes in 1 mL 1X PBS. Next, coverslips were incubated in 300 µL of primary antibody (anti-TDP-43 1:750, PTGLabs; anti-SUMO2/3 [8A2] 1:500 Sigma) diluted in blocking buffer (1.5% Triton X-100, 10% cosmic calf serum in 1X PBS) overnight at 4 °C. The next day, the coverslips were washed for 3 × 5 minutes in 1 mL of Duolink Wash Buffer A (0.01 M Tris-Base, 0.15 M NaCl, 0.05% Tween-20, pH 7.4) followed by incubation at 37°C for 1 hour in 40 µL of dilute probe mixture containing a 1:5 dilution of Duolink PLA MINUS (Sigma Aldrich, DUO82004) and PLUS probes (Sigma Aldrich, DUO82002) in antibody diluent (Sigma Aldrich, DUO82008). Duolink PLA probes were diluted in antibody diluent (Sigma Aldrich, DUO82008) at a 1:5 dilution. Coverslips were washed for 3 × 5 minutes in Duolink Wash Buffer A and then incubated in 40 µL of ligase (Sigma Aldrich, DUO82027) diluted in 1X ligation buffer (Sigma Aldrich, DUO82009) at a 1:40 dilution at 37 °C for 30 minutes. The coverslips were then washed for 3 x 5-minutes washes in Duolink Wash Buffer A and then incubated in 40 µL of polymerase (Sigma Aldrich, DUO82028) diluted 1:80 in 1X amplification buffer (Sigma Aldrich, DUO82011) at 37°C for 90 minutes. Next, the coverslips were washed 2 × 10 minutes in Duolink Wash Buffer B (0.2 M Tris-Base, 0.1 M NaCl, pH 7.5) and then again in 1 mL of Duolink Wash Buffer B diluted at 1:100 in 1X PBS for 1 minute. Coverslips were briefly air dried and then mounted on slides using Vectashield Antifade Mounting Medium with DAPI. Z-stack images were obtained on a Zeiss AxioObserverZ1 LSM800 Confocal Microscope at 63× magnification with a 5× digital zoom through a Z distance of 10-12 µm per image using optimal 0.27 µm spacing per slice with dimensions set to 512 × 512 pixels with 2X averaging per frame. ~5 images were randomly obtained around the coverslip sampling >15 cells per condition per replicate. Images were analyzed and quantified using the Spots function on the Imaris (ver. 9.9.1 Bitplane, Switzerland) software.

Behaviour testing general information

All adult behaviour testing was performed in the University of Ottawa Behaviour and Physiology Core. During behaviour testing periods the mice were minimally disturbed: food and water were only added/changed as needed and cages were changed by the experimenter with a transfer of old bedding material. Apart from nesting and Beam Break testing, all behaviour tests were performed in the light phase of the cycle. The experimenter was blinded to the genotype of the mice during behaviour testing and until the mice were end pointed. Mice were brought to the testing room to habituate in dim white light at least 30 minutes prior to commencing testing with the exceptions of nest building and fear conditioning for which there was no habituation period. The behaviour cohort consisted of males and females with 12-15 animals per sex per genotype. These mice had been backcrossed three times to C57BL/6N background. All mice used for histology, biochemistry, and RNA analyses performed the fear conditioning test 1 week prior to dissections to control for potential molecular changes induced by this behaviour test.

Developmental testing

A small battery of behaviour tests was completed at P8 and P21. At P8, these tested included (I) weight, (II) righting reflex, (III) hindlimb suspension, and (IV) forelimb suspension. (I) Weight was measured every other week by placing individual mice on a digital weigh scale. (II) Mice were placed on their back on a tabletop and timed for how long it took them to right themselves onto their paws. (III) Mice were placed facing downward in a 50mL Falcon tube (VWR 21008-940) and timed for their latency to fall. (IV) Mice were placed to grasp a wire suspended by a pencil holder and timed for latency to fall. At P21, hanging wire was performed in which the mouse was

allowed to grasp a wire 30 cm off the ground and time for its latency to fall. Each mouse completed three trials with a maximum time of 60 seconds.

Beam break

The Beam Break test was used to assess general locomotor activity and habituation to a novel cage environment. The apparatuses used to record and analyze the mice activity included the Micromax analyzer/Fusion Software (Omnitech Electronics; Columbus, OH, USA) at the 2-month time point and the Photobeam Activity System (San Diego Instruments; San Diego, CA, USA) at the 9 and 16-month timepoints. Clean cages with only a thin layer of corncob bedding, food, and water were loaded into the recording frame. Mice were singly housed in one of these cages 2 hours prior to the start of their dark cycle. For a 24-hour period at the standard 12-hour dark/light cycle, the activity of the mice was monitored by infrared beam emitters and receptors. When the test was completed, the sum of the infrared beam breaks in 5-minute, 1-hour, and 24-hour sampling bins was analyzed.

Digigait

The Digigait treadmill Imager and Analysis Software (Mouse Specifics Inc.) were used to record and analyze parameters of gait, respectively. Mice were placed on the unmoving treadmill surface and once recording was started the speed was increased to 18 cm/s with 0 degree incline. A 3 second video of each mouse walking continuously, with no stopping or starting, was captured. A mouse was excluded from the test if it was unable to walk for 3 seconds at the 18 cm/s speed. Digigait data was analyzed using FactoMinR (Lê et al., 2008) and visualized using FactoExtra (Alboukadel and Fabian, 2020) packages in R (version 4.2.3).

Fear conditioning

Contextual and cued fear conditioning were tested using a 3-day protocol to assess associative fear learning and memory. Naïve age and sex matched mice were initially used to determine the optimal shock value. On day 1 (training), the mice were placed into a Phenotyper box (Noldus Information Technology) with a grid shock floor (Med Associates). The testing room was set to 60 lux light level and 70 dB of white noise (context A). The mice were left in the apparatus for 6 minutes during which they receive 3 tone-shock pairings (30 seconds tone co-terminated with a 2 second foot shock). On day 2 (Context), the mice were placed in the same apparatus (Context A) with no tone or foot shock delivered for the 6-minute trial. On day 3 (cue), the animals were placed in the test apparatus with an altered context (context B) including red light, no white noise, vanilla scent, textured mat covering the shock floor and plastic inserts in the apparatus. The mice are allowed to explore this context with no tone for 3 minutes, and then are presented with the same tone from Day 1 for the last 3 minutes. The time freezing was analyzed with EthoVision software for all 3 testing days.

Grip strength

A grip strength meter (Chatillon DFE II, Columbus Instruments) was used to assess the maximal forelimb grip strength of the mice. The grip strength meter was rotated vertically and temporarily mounted on a flat surface prior to testing. The mouse was brought near the triangular attachment and allowed to grasp the lower bar with its forepaws. The mouse was pulled directly downward, by the tail, in one smooth motion. Each mouse was tested, and the grip strength recorded for 5 consecutive trials with a 1-minute intertrial interval. If a trial was deemed unsuccessful by the

blinded experimenter, the trial was redone. Causes of an unsuccessful trial included the mouse prematurely losing its grip on the bar or grasping the bar with its hindfoot. The average grip strength for the 5 successful trials was analyzed.

Hanging wire

The hanging wire test was used to assess muscle strength and coordination. A metal wire was secured to the top of a tall plastic box with padding on the bottom. The mouse was brought near and allowed to grasp the wire with its forepaws. A timer was started once the experimenter released the mouse to allow it to hang freely. When the mouse fell from the wire the time was recorded. Mice had three trials and were allowed to hang for a maximum of 600 seconds with a 60 second intertrial interval in between. If a mouse hung for 600 seconds, it did not complete any additional trials. The maximum hanging time from the trials was used for analysis.

Light/dark box

The light dark paradigm was used to assess anxiety-like or disinhibited, exploratory behaviour. The testing apparatus (Med Associates) has two equal sized rectangular compartments the mouse can move freely between. The one is fully illuminated whereas the other is covered by a black plastic insert. The position of the mouse in the apparatus testing field is recorded using infrared beams. To start the test, the mice are placed into the lit side and allowed to explore for 10 minutes. The time spent in each compartment and number of entries into each compartment was recorded and analyzed by Activity Monitor software (Med Associates).

Marble burying

The marble burying assay can assess the motor function required to bury objects as well as cognitive changes related to apathy or perseverance. Cages were filled with 10cm of fresh corncob bedding and one mouse was placed to habituate for 5 minutes. After habituation, 20 glass marbles were laid out evenly in a 4 by 5 pattern. The mouse was returned to the cage and left alone for 30 minutes in 60 lux light. With the completion of the trial, the number of marbles buried by at least two thirds was scored by a blinded experimenter.

Nest building

Nest building is an innate behaviour of mice in their daily lives. This complex behaviour requires both executive planning and sensorimotor coordination. Directly following Beam Break testing, a single square nestlet (5 cm² cotton pad) was placed in each Beam Break cage for 16 – 18 hours, of which 12 of these hours was during the dark cycle. At the end of the test, images were taken of the nests and scored blindly for quality as previously described on a scale from 1-5.

Open field

The open field test was used to assess anxiety and locomotor activity in a novel environment. The apparatus consists of white plastic square arenas measuring 45cm on each side. The mice were placed in a corner of the arena and allowed to freely explore for 10 minutes with light levels at 300 lux. The distance travelled, time spent in the corners and center of the field was recorded and analyzed by EthoVision software (Noldus Information Technology).

Rotarod

The rotarod (IITC Life Science, Ugo Basile) was used to test the motor performance of mice including coordination and resistance to fatigue. Mice were placed in the stationary rotarod bar for 10 seconds before the rotarod program was initiated. The bar accelerated from 4 rpm to 40 rpm for 5 minutes and the latency to fall for each mouse was recorded. The time was stopped when the mouse fell from the bar or rotated passively. Mice were completed four trials per day, with a 10-minute intertrial interval in their home cage, for 3 consecutive days.

Spontaneous Y-maze

The spontaneous Y-Maze test was used to assess spatial working memory. The Y-shaped maze has three identical arms at 120° around a center point triangle. The mice were placed in the center point and allowed to freely explore the arms for 8 minutes. The movement of the mouse was tracked including the sequence of arm entries by EthoVision (Noldus Information Technology). An alternation is defined as the mouse making consecutive, sequential entries into each of the three arms without revisiting an arm. The alternation index was calculated as (number of alternations/(total number of arm entries minus two)) and reported as a percent.

Tail suspension

The automated Tail Suspension apparatus (Med Associates) was used to assess apathetic-like behaviours of the mice. Mice were taped and suspended by the tail to a vertical steel bar which measures strain gauge as the mouse moves during a 6-minute trial. The cumulative time spent immobile, hanging passively (below lower threshold) was measured by the Tail Suspension software.

Three chamber sociability

The Three Chamber test was conducted to measure the sociability of mice when allowed to interact with a novel mouse or a similarly sized inanimate object. The testing apparatus is a 19 x 45 cm plastic box divided into three equal chambers with clear plastic wall dividers. The two external chambers each have a single weighted metallic mesh pencil holder and the central chamber is empty. The mice are habituated to the apparatus for 5 minutes by being placed in the central chamber and allowed to freely explore and enter all chambers. The mouse has a 5-minute intertrial interval in its home cage. In the test trial, a sex and age matched wild-type mouse (social target) is placed beneath one mesh pencil holder, and an inanimate plastic toy (non-social target) is placed beneath the other. To commence the test trial, the mouse is placed in the central chamber. The time spent in each chamber and interacting with the social or non-social target is recorded and analyzed by EthoVision software (Noldus Information Technology).

Tube test of social dominance

Tube test was used to assess social dominance and within cage social hierarchy. Cage mates were paired against each other for testing in a round-robin design. The tube test was conducted on a flat tabletop, which the mice were allowed to run around on for 5 minutes prior to commencing the testing. One foot of vinyl tubing was used. Mice were habituated to the tube prior to testing on the same day by encouraging them to run through the empty tube from either side 5 times. A blinded experimenter placed a mouse on either end of the tube, and released their tails when they completely entered the tube. The first mouse to step its hind paws out of the tube lost the battle. The battle was redone if after 2 minutes no mouse had won.

Collection and preparation of mouse tissue

Mice were harvested for both histology and biochemistry/molecular analyses to reduce total animal numbers. Mice were first acclimated for 2 hours prior to dissection to help control for activity-induced changes and all dissections were performed at zeitgeber hours ZT6 to ZT11 to control for potential circadian affects. Mice were randomly dissected by cage to limit batch effects and additional stress. Mice were anesthetized using isoflurane inhalation, blood was collected, and then mice were sacrificed via decapitation and the brains were quickly isolated, weighted, then cut into 2 hemispheres where the left hemisphere was drop fixed in 10% buffered formalin and the right hemisphere was dissected by region and flash frozen on dry ice. Spinal cords were removed using hydraulic extrusion and the lumbar spinal cord was isolated. The lumbar enlargement was isolated for histology whereas the remaining lumbar spinal cord was used for molecular analysis. Tibialis anterior and soleus muscles were isolated and drop fixed in 10% buffered formalin for imaging studies and gastrocnemius muscle was flash frozen.

Immunofluorescence analysis in mouse tissue

Tissue was collected as described in *Collection and preparation of mouse tissue*. Following fixation for 48 hours, spinal cord samples were transferred to a 70% ethanol solution and sent to the Louise Pelletier Histology Core facility at the University of Ottawa for paraffin embedding and microtome sectioning. The samples were sectioned at a thickness of 5 μm . Serial sectioning was performed at four levels within the tissue each separated by 40 μm . For staining, slide-mounted sections were deparaffinized in 100% xylenes (Fisher Scientific X3P-1GAL) for 10 minutes, then transferred into a second container of fresh 100% xylenes for an additional 10 minutes. The slides

were then rehydrated in descending ethanol solutions: two 5 minutes treatments in 100% ethanol, 5 minutes in 70% ethanol, and 5 minutes in 50% ethanol. The slides were then immersed in 1X PBS for at least 5 minutes to rinse residual ethanol. Antigen was performed by placing slides in 1X sodium citrate buffer (2.94g sodium citrate 0.5mL Tween-20 in 1L 1X PBS, pH6) at 95°C for 30 minutes then rinsed in 1X PBS. Tissue sections were then blocked in blocking buffer containing 1% Triton-X and serum (5% horse serum, 5% cosmic calf serum, or 1% bovine serum albumin) in 1X PBS for 2 hours at room temperature. Next the tissue sections were incubated with primary antibody (table S5) overnight at 4°C. The next day, the sections were washed twice in PBS 1X + 0.1% Triton-X for 5 minutes each and then 3X in 1X PBS for 5 minutes each. The slides were then incubated in secondary antibody at room temperature for 2 hours. The slides were then washed in 1X PBS + 0.1% Triton-X for 5 x 5-minutes, before mounting with #1.5 coverslips (Thermo Fisher Scientific 12-544E) and fluorescent mounting media (Dako S3023). The coverslips were sealed with clear nail polish and allowed to dry before imaging. Images of each ventral horn were taken on a Zeiss Axio Imager 2 at 20X magnification. Cells were counted manually per image using Fiji (ImageJ).

Neuromuscular junction analysis

Tissue was collected as described in *Collection and preparation of mouse tissue*. After 24 hours of fixation, tibialis anterior muscles were stored in a solution of 1X PBS. Muscle bundles were carefully teased apart from the tissue into 4-5 bundles with forceps. The muscle bundles were transferred to 6-well plate cell culture plate containing 1% Triton-X in 1X PBS overnight at 4°C under gentle agitation for permeabilization. The next day, the muscle bundles were washed three times in 1X PBS for 5 minutes at room temperature. They were placed in a blocking buffer solution

of 4% bovine serum albumin and 1% Triton-X in 1X PBS at 4°C overnight. The following day, the muscles were incubated in primary antibody (BTX and NfL/SV2 cocktail, see table S5) diluted in blocking buffer solution overnight at 4°C. The next day, the muscle bundles were washed with 1X PBS three times for 5 minutes and incubated with secondary antibody diluted in blocking buffer solution at room temperature for 2 hours. The muscle bundles were then washed 3 times in 1X PBS for 5 minutes. The bundles were then placed on slides, mounted with VECTASHIELD® HardSet™ Antifade Mounting Medium (#H-1400), and #1.5 coverslips placed above the muscle bundles. The slides were left at room temperature overnight in the dark to allow the mounting media to cure. Images were taken on a Zeiss LSM800 Confocal Microscope at 20X through a Z-stack with optimal spacing (0.61 μm). Images were taken throughout the entirety of the muscle tissue such that at least 80 neuromuscular junctions per animal were captured. Images were scored manually per image using Fiji (ImageJ). See Fig. S8E for scoring examples.

SUMOylation analysis in human brain tissue

20 mg of frozen human brain tissue was homogenized in 300 μL of RIPA buffer supplemented with protease inhibitor, phosphatase inhibitor, and 50mM N-Ethylmaleimide and vortexed every 5 minutes for 20 minutes while incubating on ice. The samples were centrifuged at 21000G for 20 minutes at 4°C and the supernatant (RIPA soluble fraction) was carefully removed. 4X Laemmli buffer was added and samples were boiled for 5 minutes at 95 °C then stored overnight at 4 °C. The remaining pellet after the centrifugation step post-lysis was washed in 1mL RIPA buffer with vortexing for 10 seconds followed by centrifugation at ~21000G for 20 minutes at 4 °C. The supernatant was carefully removed, and the pellet was resuspended in 300 μL of 8M UREA in PBS with 10mM Tris-HCl pH 7.4. Samples were vortexed and left to solubilize overnight at room

temperature before 4X Laemmli was added prior to Western Blot analysis. RIPA and UREA samples were randomly loaded onto gels (separated by fraction) with 4 control samples consistently loaded (aged 20-30) on each gel for normalization. Western blot was performed as described above. Samples were standardized to ponceau signal then normalized to control samples.

Collection and preparation of FFPE human brain tissue

Frontal cortex tissues were collected antemortem from three sporadic ALS/FTLD patients (a 74-year-old male, 67-year-old female and 59-year-old female) obtained through the ALS Clinic at Sunnybrook Health Sciences Centre, Toronto. ALS was diagnosed using the revised El Escorial Criteria (Brooks et al., 2000) and informed consent was obtained with approval from the local ethical review board. The presence of TDP-43 proteinopathy within the frontal cortex was verified through immunohistochemical labeling using rat anti-phosphorylated TDP-43 (p409/410) antibody on formalin fixed paraffin embedded sections. Genetic analyses confirmed absence of mutations in key ALS/FTD-associated genes: *C9orf72*, *SOD1*, *FUS* and *TARDBP*.

Proximity ligation assay in human FFPE tissue

Formalin fixed paraffin-embedded human brain sections (5 µm sections) were deparaffinized using two 10-minute washes with xylenes, two 5-minute washes with 100% ethanol, one 5-minute wash with 70% ethanol, and one 5-minute wash with 50% ethanol. Deparaffinized sections then underwent sodium citrate antigen retrieval (10mM sodium citrate, 0.05% Tween-20, pH 6) for 2 hours at 80°C. Sections were then blocked with Duolink blocking buffer (Sigma Aldrich, DUO82007) for 1 hour at room temperature. Next, sections were incubated with primary antibodies (anti-TDP-43 1:750, PTGLabs; anti-SUMO2/3 [8A2] 1:500 Sigma) diluted in blocking

buffer (1.5% Triton X-100, 10% cosmic calf serum in 1X PBS) overnight at 4°C. The following day, the sections were washed in Duolink Wash Buffer A (0.01M Tris-Base, 0.15M NaCl, 0.05% Tween-20, pH 7.4), followed by incubation in Duolink PLA MINUS (Sigma Aldrich, DUO82004) and PLUS probes (Sigma Aldrich, DUO82002) at 37°C for 1 hour. Duolink PLA probes were diluted in antibody diluent (Sigma Aldrich, DUO82008) at a 1:5 dilution. Sections were washed in Duolink Wash Buffer A and then incubated in ligase (Sigma Aldrich, DUO82027) at 37°C for 30 minutes. Ligase was diluted in 1X ligation buffer (Sigma Aldrich, DUO82009) at a 1:40 dilution. Then, the sections were washed in Duolink Wash Buffer A and then incubated in polymerase (Sigma Aldrich, DUO82028) at 37°C for 90 minutes. Polymerase was diluted in 1X amplification buffer (Sigma Aldrich, DUO82011) at a 1:80 solution. The sections were then washed in Duolink Wash Buffer B (0.2M Tris-Base, 0.1M NaCl, pH 7.5) and then again in Duolink Wash Buffer B diluted at 1:1000. Finally, sections were incubated with DAPI at room temperature for 15 minutes. DAPI was diluted at 1:1000 in PBS. Fluorescent mounting media (Dako, S3023) and #1.5 coverslips were then placed over the sections and sealed using clear nail polish. Images were taken on a Zeiss LSM800 Confocal Microscope. 5 images per tissue were randomly collected from grey matter at 63X magnification and 2X digital zoom through a Z stack of 10-12 μm with optimal spacing (0.27 μm) at a resolution of 512 x 512 pixels. PLA foci were manually counted per image using Fiji (ImageJ).

Statistical Analyses

Statistical tests were performed using PRISM 10.2.2. Test type was selected based on the number of comparisons made, repeated measures, and gaussian distribution of the data. Levels of statistical significance are indicated in figure legends.

2.7 Acknowledgments

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2.8 Supplementary Information

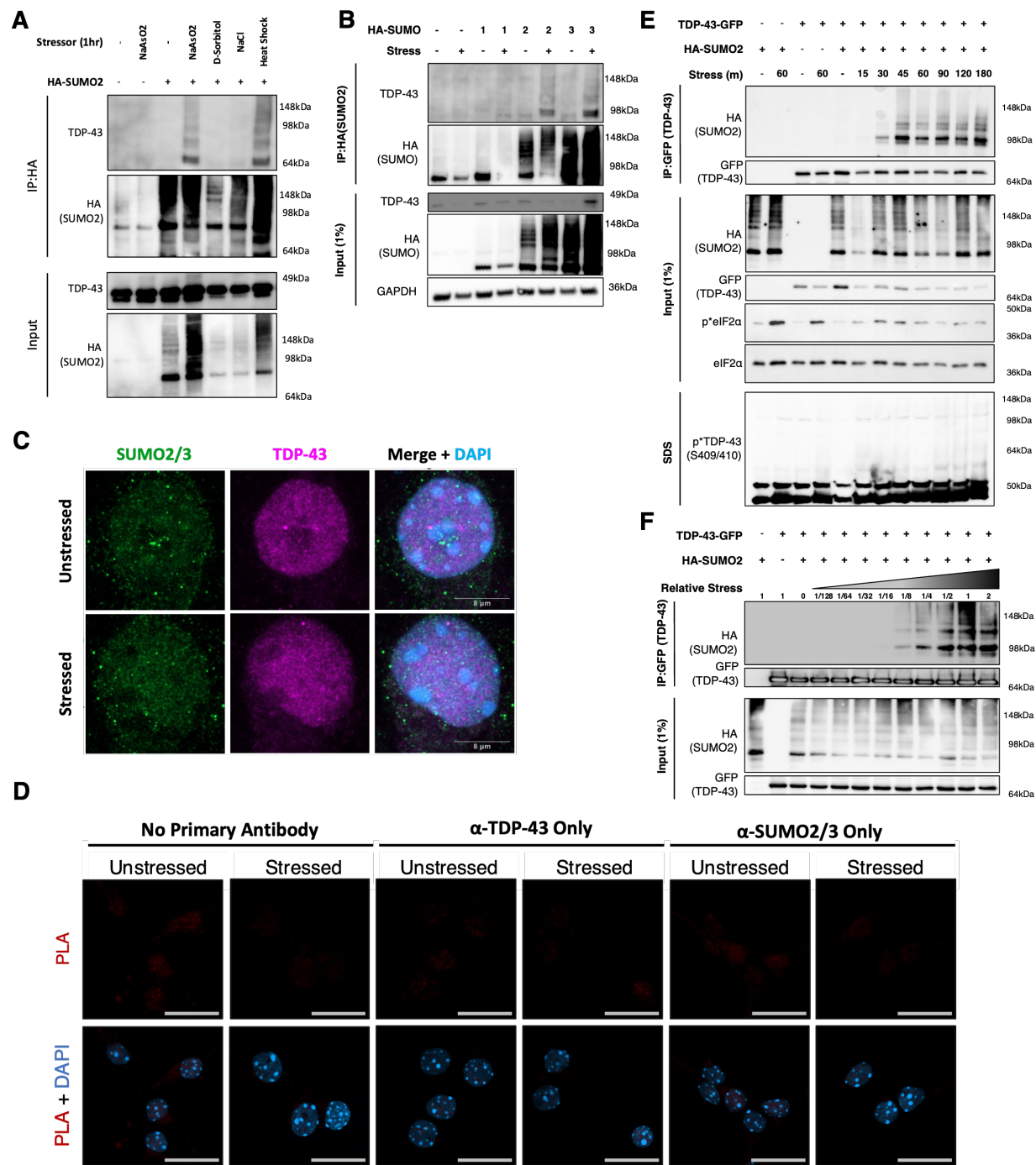


Fig. S1: Characterizing stress responsive TDP-43 SUMOylation.

(A) Representative western blot from SUMOylation assays testing whether various stressors that cause TDP-43 aggregation induce TDP-43 SUMOylation: NaAsO₂ (250 μ M), D-Sorbitol (400 mM), NaCl (300 mM), Heat Shock (42 °C). **(B)** Representative western blot from SUMOylation assays testing the selectivity of SUMO paralogs to SUMOylate TDP-43 in response to 1 hour sodium arsenite (250 μ M) stress. **(C)** Representative images of SUMO2/3 and TDP-43 in the nucleus of mouse cortical motor neurons (7 DIV) in unstressed and 1 hour sodium arsenite (250 μ M) stressed conditions. **(D)** Representative control imaged from Proximity Ligation Assay (PLA) in mouse primary cortical neurons. Scalebar = 20 μ m. (N = 4). **(E)** Representative western blot and quantification from GFP-Trap SUMOylation assays testing the kinetics of TDP-43 SUMOylation in response to sodium arsenite stress (250 μ M) and its relationship to insoluble phosphorylated TDP-43. (N = 3) **(F)** Representative dose-response assay to detect TDP-43 SUMOylation after 1 hour of sodium arsenite stress (1 = 250 μ M, 1/2 = 125 μ M, 1/4 = 62.5 μ M, etc.)

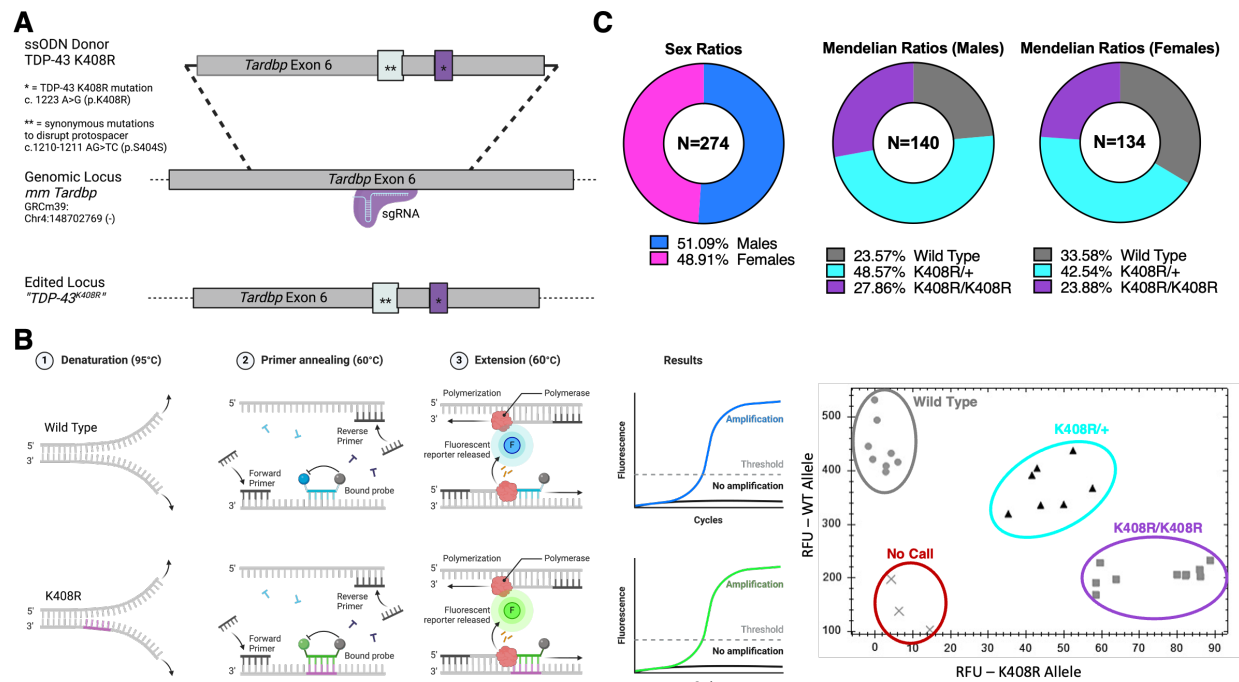
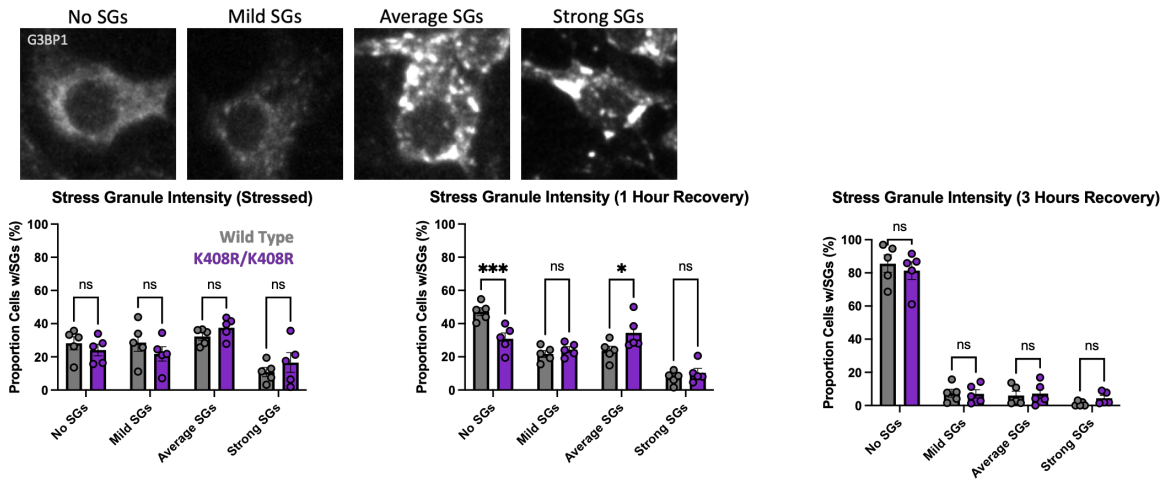
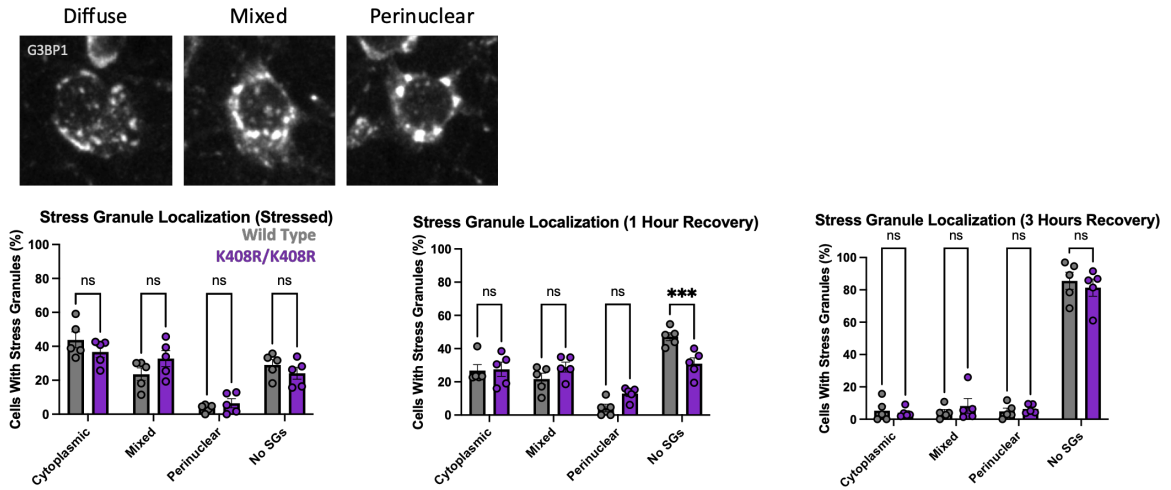
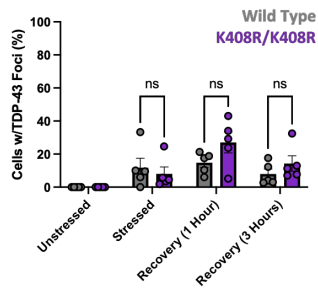


Fig. S2: Generation of the TDP-43^{K408R} mouse line.

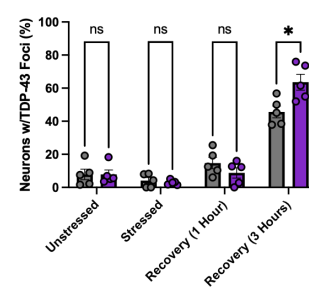
(A) Schematic of the knock-in approach to edit the endogenous *Tardbp* to express TDP-43^{K408R}. (B) Schematic of LNA probe based genotyping approach and allelic discrimination assay to genotype TDP-43^{K408R} mouse line. (C) Sex and mendelian ratios of F3 generation TDP-43^{K408R} mice from K408R/+ and K408R/+ breeding pairs used to generate behaviour and histology cohorts. Observed vs Expected statistical analysis assuming an expected 1:1 ratio sex ratio and 1:2:1 mendelian ratio.

A**B****C**

TDP-43 Foci in Stress Granule Positive Neurons



TDP-43 Foci in Stress Granule Negative Neurons

**D**

Average Number of TDP-43 Nuclear Foci

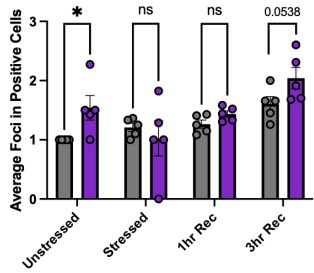
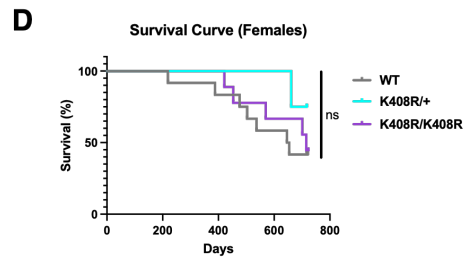
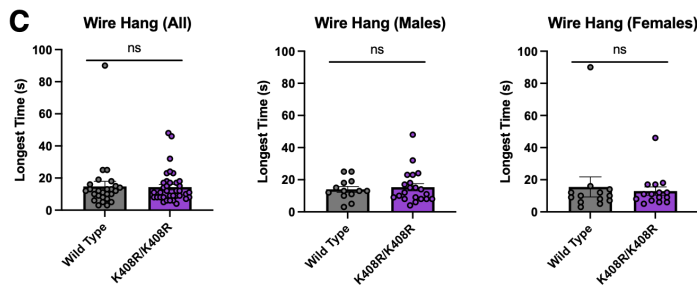
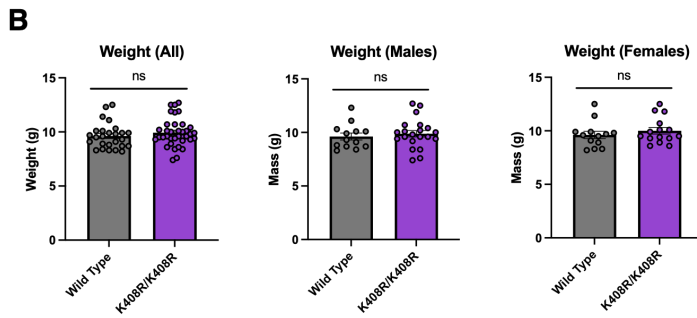
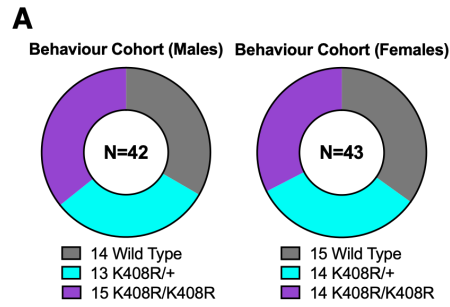


Fig. S3: Characterizing the stress response in TDP-43^{K408R} primary cortical neuron cultures.

(A) Classification and quantification of G3BP1 stress granule (SG) intensity in response to 1 hour sodium arsenite stress (250 μ M) and recovery. (N = 5 per genotype) 2-Way ANOVA with Tukey's multiple comparisons analysis. * $p < 0.05$, *** $p < 0.0005$. **(B)** Quantification of the localization of G3BP1 stress granules (SGs) in response to 1 hour sodium arsenite stress (250 μ M) and recovery. (N = 5 per genotype) 2-Way ANOVA with Tukey's multiple comparisons analysis. *** $p < 0.0005$. **(C)** Quantification of the percentage of neurons with positive TDP-43 nuclear foci in G3BP1 stress granule positive and G3BP1 stress granule negative neurons in response to 1 hour sodium arsenite stress (250 μ M) and recovery. (N = 5 per genotype) 2-Way ANOVA with Tukey's multiple comparisons analysis. *** $p < 0.0005$. **(D)** Quantification of the average number of TDP-43 nuclear foci in neurons positive for TDP-43 nuclear foci in response to 1 hour sodium arsenite stress (250 μ M) and recovery. (N = 5 per genotype) 2-Way ANOVA with Tukey's multiple comparisons analysis. * $p < 0.05$.



Legend: Wild Type K408R/K408R

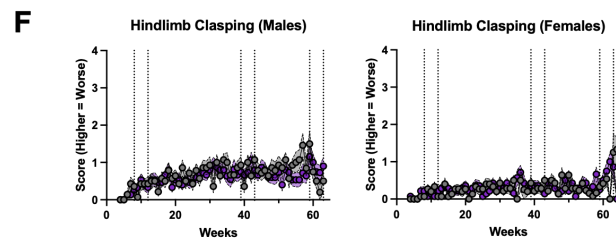
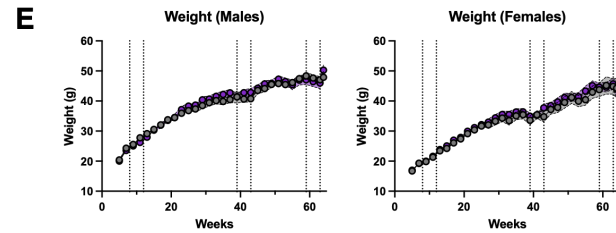


Fig. S4: General wellness of TDP-43^{K408R} mice during development and aging.

(A) Genotype and sex composition of mice subject to behaviour testing. **(B)** Weight of mice at P21. Unpaired T-test (Males); Mann-Whitney test (Females and All) **(C)** Quantification of the duration hanging on a wire at P21. Mann-Whitney test (Males, Females, and All). **(D)** Survival curve for female TDP-43^{K408R} mice (Males found in Fig. 5d). Curve comparisons analyzed using Log-Rank test and Gehan-Breslow-Wilcoxon test. **(E)** Weights of male and female TDP-43^{K408R} mice from 3 weeks to 64 weeks of age. Mixed-effects analysis. **(F)** Hindlimb clasping scores of male and female TDP-43^{K408R} mice from 3 weeks to 64 weeks of age. Mixed-effects analysis. See table S2 for raw data details on statistical tests.

Fig. S5: Characterizing the cognitive and social behaviour of TDP-43^{K408R} mice.

Quantification of cognitive and social behaviours in the **(A)** Light/Dark Box, **(B)** Y-maze, **(C)** 3 Chamber Social Interaction, **(D)** Marble Burying, and **(E)** Tail Suspension behaviour tasks at 2, 9, and 16 months of age (2MO, 9MO, and 16MO, respectively) in male and female TDP-43^{K408R} mice. All behaviour tests were analyzed with a Mixed-Effects Model with Tukey's multiple comparison analysis, see table S2.

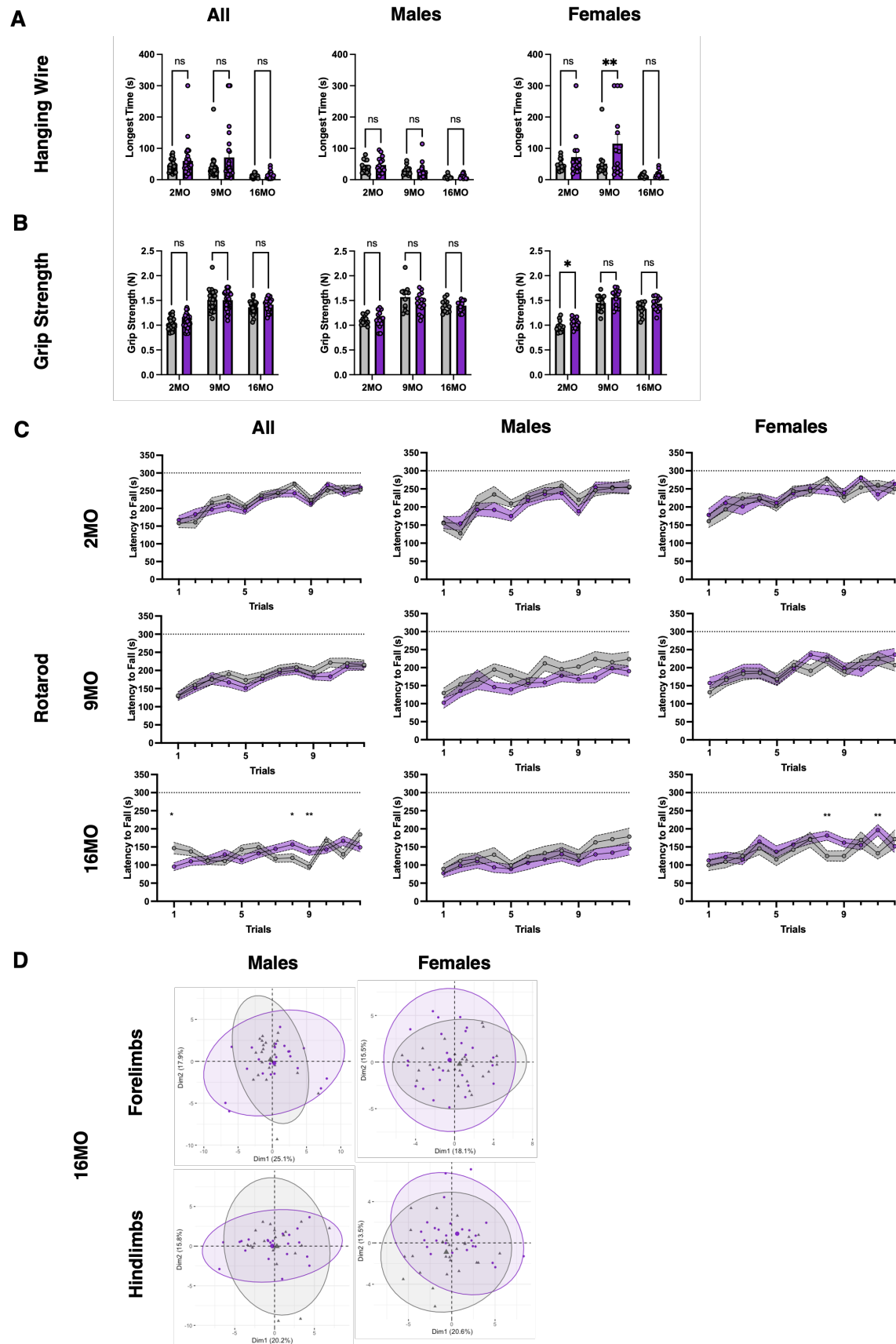


Fig. S6: Characterizing the motor behaviour of TDP-43^{K408R} mice.

Quantification of motor abilities in the **(A)** Hanging Wire, **(B)** Grip Strength, and **(C)** Rotarod behaviour tasks at 2, 9, and 16 months of age (2MO, 9MO, and 16MO, respectively) in male and female TDP-43^{K408R} mice. **(D)** Digigait behaviour tasks at 16 months of age in male and female TDP-43^{K408R} mice. Behaviour tests were analyzed with 2-Way ANOVA or Mixed-Effects Models, see table S2.

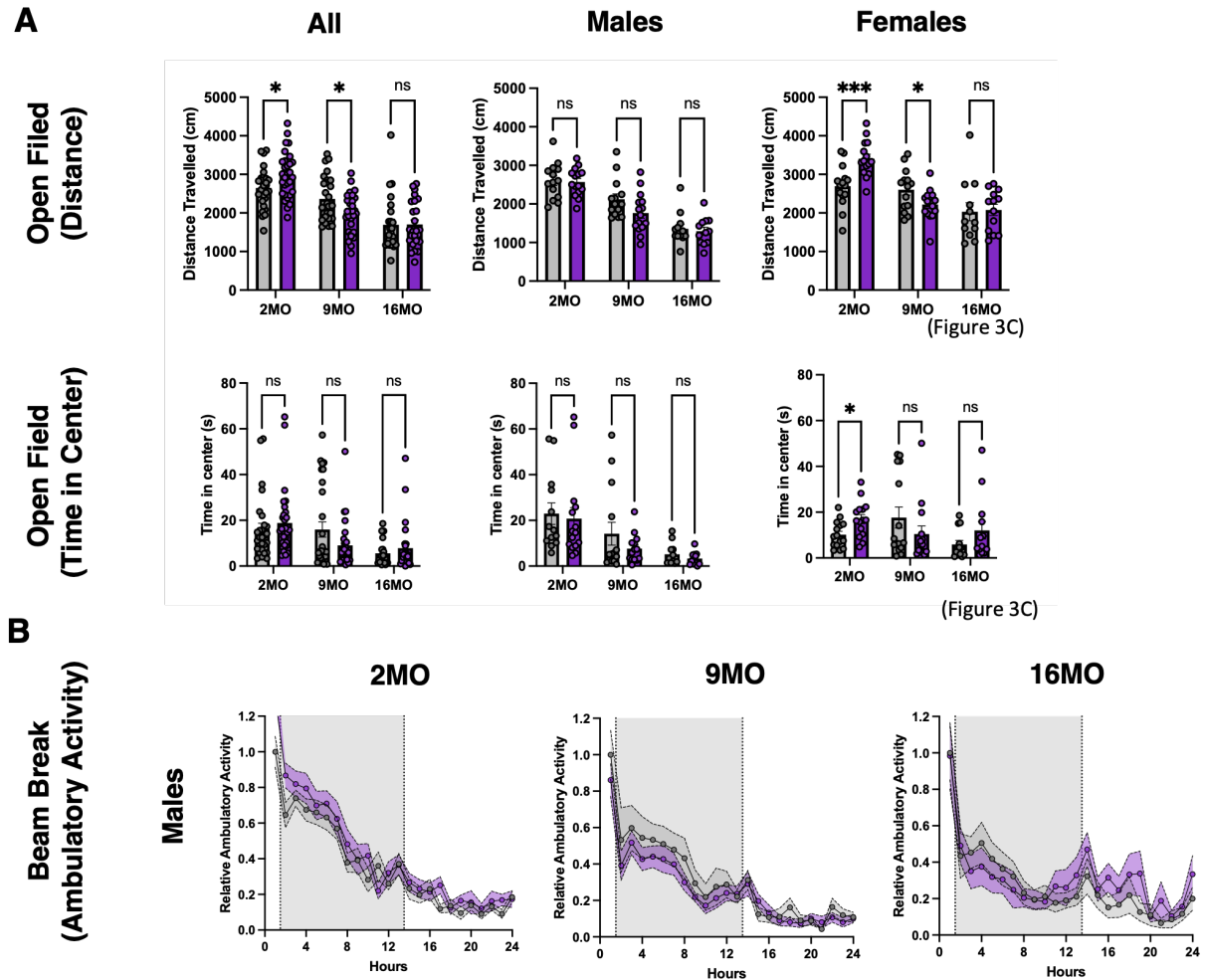


Fig. S7: Characterizing the activity of TDP-43^{K408R} mice.

Quantification of **(A)** total distance traveled (cm) and time in center (s) during the Open Field behaviour test and **(B)** the relative ambulatory activity (males) of TDP-43^{K408R} mice at 2, 9, and 16 months of age (2MO, 9MO, and 16MO, respectively). Behaviour tests were analyzed with 2-Way ANOVA or Mixed-Effects models, see table S2.

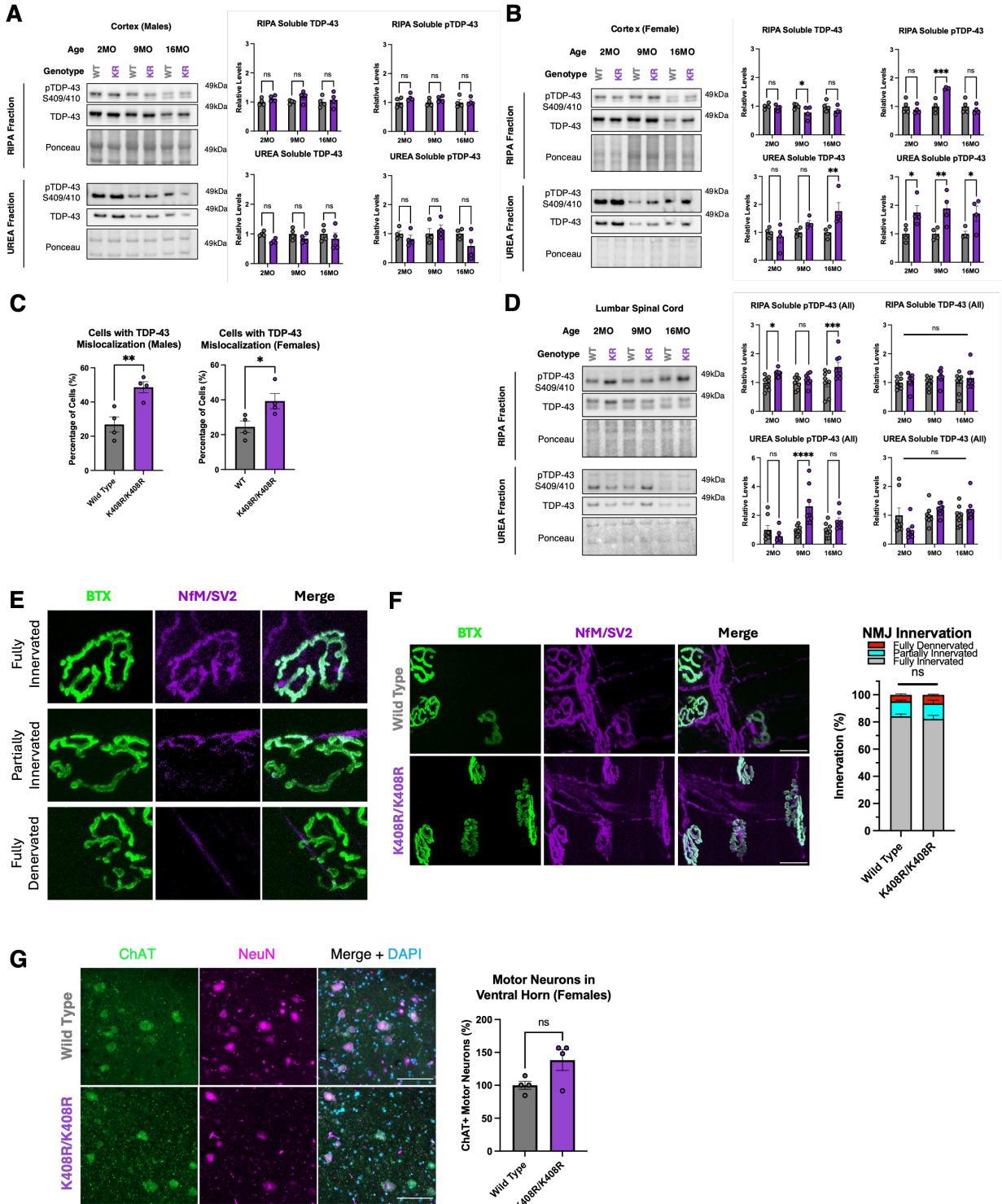


Fig. S8: Characterizing the molecular and histological effects of blocking TDP-43 SUMOylation in vivo.

(A) Representative western blot and quantification of TDP-43 and phosphorylated TDP-43 in the RIPA and UREA fractions of the cortex from male mice at 2, 9, and 16 months of age (2MO, 9MO, and 16MO, respectively. N = 4 per genotype) 2-Way ANOVA with Fisher's LSD test. **(B)** Representative western blot and quantification of TDP-43 and phosphorylated TDP-43 in the RIPA and UREA fractions of the cortex from male mice at 2, 9, and 16 months of age (2MO, 9MO, and 16MO, respectively). (N = 4 per genotype) 2-Way ANOVA with Fisher's LSD test, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. **(C)** Representative western blot and quantification of TDP-43 and phosphorylated TDP-43 in the RIPA and UREA fractions of the lumbar spinal cord at 2, 9, and 16 months of age (2MO, 9MO, and 16MO, respectively. N = 8 per genotype; 4 per sex/genotype) 2-Way ANOVA with Fisher's LSD test, * $p < 0.05$, *** $p < 0.0005$, **** $p < 0.0001$. **(D)** Quantification of TDP-43 mislocalization in the lumbar spinal cord of 9-month-old from Fig. 5a split by sex. Each datapoint represents the average of 4 serial sections 40 μm apart from an individual mouse. Unpaired t-test. * = $p < 0.05$, ** = $p < 0.005$. **(E)** Representative images and quantification of ChAT+ motor neurons in the ventral horn of the lumbar spinal cord of male and female mice. Each datapoint is the average of 4 serial sections spaced 40 μm apart through the lumbar enlargement of the lumbar spinal cord. Unpaired t-test, ** $p < 0.005$. **(F)** Example images of neuromuscular junctions (NMJs) for scoring criteria to assess fully innervated, partially innervated, and fully denervated NMJs.

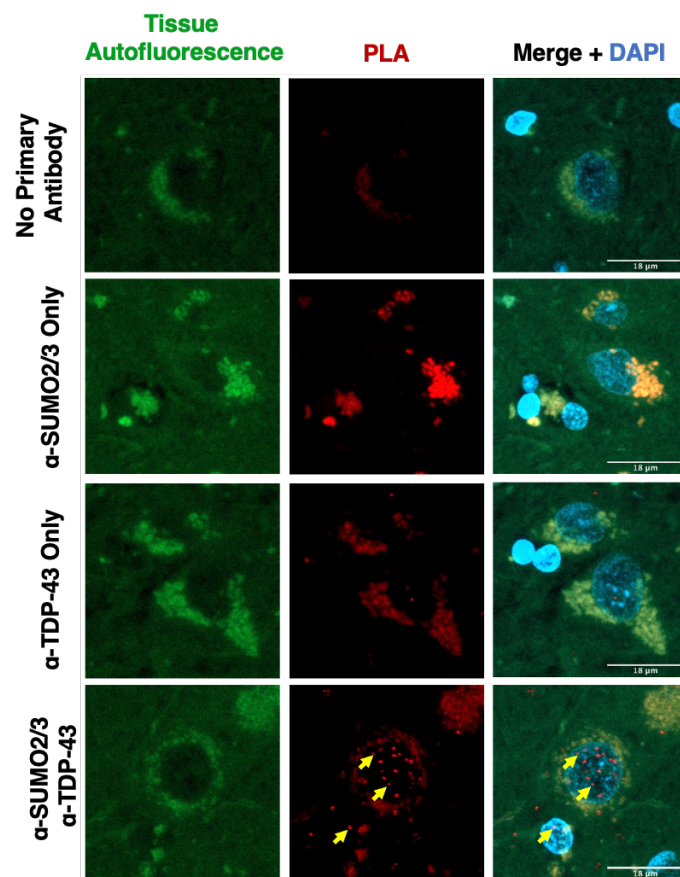


Fig. S9: Optimization of proximity ligation assay in human tissue.

Representative images of proximity ligation assay (PLA) between TDP-43 and SUMO2/3 in human PPFE hippocampus tissue sections (5 μ m). Yellow arrows indicate TDP-43:SUMO2/3 interactions where the signal specific to the PLA channel and not identified in the autofluorescence channel. Scalebar = 18 μ m.

Table S1: K408 Conservation Analysis

Due to size and complexity, table can be found online:

<https://www.biorxiv.org/content/10.1101/2024.04.12.589206v1.supplementary-material>

Table S2: Behaviour Analysis

Due to size and complexity only a summary table of the full table contents is shown below.

The full table can be found online:

<https://www.biorxiv.org/content/10.1101/2024.04.12.589206v1.supplementary-material>

Sheet Number	Data Analyzed	Age	N (Wild Ty) N (K408R/K408R)		Sheet Number	Statistical Test	Post Hoc
Sheet 1	Cumulative Barbering (Females)	3-18wk			Sheet 1	2 Way ANOVA	Tukey's
Sheet 2	Development Weight (Males)	P10-11	13	21	Sheet 2	Unpaired T-test	NA
Sheet 3	Development Weight (Females)	P10-11	13	15	Sheet 3	Mann-Whitney Test	NA
Sheet 4	Development Weight (All)	P10-11	26	36	Sheet 4	Mann-Whitney Test	NA
Sheet 5	Development Wire Hang (Males)	P10-11	13	21	Sheet 5	Mann-Whitney Test	NA
Sheet 6	Development Wire Hang (Females)	P10-11	13	15	Sheet 6	Mann-Whitney Test	NA
Sheet 7	Development Wire Hang (All)	P10-11	26	36	Sheet 7	Mann-Whitney Test	NA
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 8	Open Field Distance Traveled (Males)	9MO	14	15	Sheet 8	Mixed-Effects Model (REML)	Tukey's
		16MO	12	12		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 9	Open Field Distance Traveled (Females)	9MO	15	15	Sheet 9	Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's
		2MO	29	30		Mixed-Effects Model (REML)	Tukey's
Sheet 10	Open Field Distance Traveled (All)	9MO	29	30	Sheet 10	Mixed-Effects Model (REML)	Tukey's
		16MO	25	25		Mixed-Effects Model (REML)	Tukey's
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 11	Open Field Time in Center (Males)	9MO	14	15	Sheet 11	Mixed-Effects Model (REML)	Tukey's
		16MO	12	12		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 12	Open Field Time in Center (Females)	9MO	15	15	Sheet 12	Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's
		2MO	29	30		Mixed-Effects Model (REML)	Tukey's
Sheet 13	Open Field Distance Traveled (All)	9MO	29	30	Sheet 13	Mixed-Effects Model (REML)	Tukey's
		16MO	25	25		Mixed-Effects Model (REML)	Tukey's
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 14	Nesting (Males)	9MO	13	15	Sheet 14	Mixed-Effects Model (REML)	Tukey's
		16MO	12	12		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 15	Nesting (Females)	9MO	15	15	Sheet 15	Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's
		2MO	29	30		Mixed-Effects Model (REML)	Tukey's
Sheet 16	Nesting (All)	9MO	28	30	Sheet 16	Mixed-Effects Model (REML)	Tukey's
		16MO	25	25		Mixed-Effects Model (REML)	Tukey's
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 17	Y-Maze (Males)	9MO	14	15	Sheet 17	Mixed-Effects Model (REML)	Tukey's
		16MO	12	11		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 18	Y-Maze (Females)	9MO	13	14	Sheet 18	Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's
		2MO	29	30		Mixed-Effects Model (REML)	Tukey's
Sheet 19	Y-Maze (All)	9MO	27	29	Sheet 19	Mixed-Effects Model (REML)	Tukey's
		16MO	25	24		Mixed-Effects Model (REML)	Tukey's
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 20	Marble Burying (Males)	9MO	14	15	Sheet 20	Mixed-Effects Model (REML)	Tukey's
		16MO	12	12		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 21	Marble Burying (Females)	9MO	14	15	Sheet 21	Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's
		2MO	29	30		Mixed-Effects Model (REML)	Tukey's
Sheet 22	Marble Burying (All)	9MO	28	30	Sheet 22	Mixed-Effects Model (REML)	Tukey's
		16MO	25	25		Mixed-Effects Model (REML)	Tukey's
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 23	Hanging Wire (Males)	9MO	14	15	Sheet 23	Mixed-Effects Model (REML)	Tukey's
		16MO	12	12		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 24	Hanging Wire (Females)	9MO	14	14	Sheet 24	Mixed-Effects Model (REML)	Tukey's
		16MO	10	12		Mixed-Effects Model (REML)	Tukey's
		2MO	29	30		Mixed-Effects Model (REML)	Tukey's
Sheet 25	Hanging Wire (All)	9MO	28	29	Sheet 25	Mixed-Effects Model (REML)	Tukey's
		16MO	22	24		Mixed-Effects Model (REML)	Tukey's
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 26	Grip Strength (Males)	9MO	14	15	Sheet 26	Mixed-Effects Model (REML)	Tukey's
		16MO	12	12		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 27	Grip Strength (Females)	9MO	14	14	Sheet 27	Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's
		2MO	29	30		Mixed-Effects Model (REML)	Tukey's
Sheet 28	Grip Strength (All)	9MO	28	29	Sheet 28	Mixed-Effects Model (REML)	Tukey's
		16MO	25	25		Mixed-Effects Model (REML)	Tukey's
		2MO	14	15		Mixed-Effects Model (REML)	Tukey's
Sheet 29	Light/Dark Box (Males)	9MO	13	14	Sheet 29	Mixed-Effects Model (REML)	Tukey's
		16MO	12	14		Mixed-Effects Model (REML)	Tukey's
		2MO	15	15		Mixed-Effects Model (REML)	Tukey's
Sheet 30	Light/Dark Box (Females)	9MO	13	14	Sheet 30	Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's

Sheet 31	Light/Dark Box (All)	2MO	29	30	Sheet 31	Mixed-Effects Model (REML)	Tukey's
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		16MO	25	27		Mixed-Effects Model (REML)	Tukey's
Sheet 32	Tail Suspension (Males)	2MO	14	15	Sheet 32	Mixed-Effects Model (REML)	Tukey's
		9MO	13	15		Mixed-Effects Model (REML)	Tukey's
		16MO	11	12		Mixed-Effects Model (REML)	Tukey's
Sheet 33	Tail Suspension (Females)	2MO	15	15	Sheet 33	Mixed-Effects Model (REML)	Tukey's
		9MO	14	15		Mixed-Effects Model (REML)	Tukey's
		16MO	12	13		Mixed-Effects Model (REML)	Tukey's
Sheet 34	Tail Suspension (All)	2MO	29	30	Sheet 34	Mixed-Effects Model (REML)	Tukey's
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		16MO	23	25		Mixed-Effects Model (REML)	Tukey's
Sheet 35	Social Interaction (Males)	2MO	14	15	Sheet 35	Mixed-Effects Model (REML)	Tukey's
		9MO	14	15		Mixed-Effects Model (REML)	Tukey's
		16MO	12	12		Mixed-Effects Model (REML)	Tukey's
Sheet 36	Social Interaction (Females)	2MO	15	15	Sheet 36	Mixed-Effects Model (REML)	Tukey's
		9MO	14	14		Mixed-Effects Model (REML)	Tukey's
		16MO	13	13		Mixed-Effects Model (REML)	Tukey's
Sheet 37	Social Interaction (All)	2MO	29	30	Sheet 37	Mixed-Effects Model (REML)	Tukey's
		9MO	28	29		Mixed-Effects Model (REML)	Tukey's
		16MO	25	25		Mixed-Effects Model (REML)	Tukey's
Sheet 38	Beam Break Normalized Ambulatory Activity (Males)	2MO	15	14	Sheet 38	2 Way ANOVA	Tukey's
Sheet 39	Beam Break Normalized Ambulatory Activity (Females)	2MO	14	15	Sheet 39	2 Way ANOVA	Tukey's
Sheet 40	Beam Break Normalized Ambulatory Activity (Females)	9MO	14	14	Sheet 40	2 Way ANOVA	Tukey's
Sheet 41	Beam Break Normalized Ambulatory Activity (Males)	9MO	14	15	Sheet 41	2 Way ANOVA	Tukey's
Sheet 42	Beam Break Normalized Ambulatory Activity (Females)	16MO	13	12	Sheet 42	2 Way ANOVA	Tukey's
Sheet 43	Beam Break Normalized Ambulatory Activity (Males)	16MO	11	12	Sheet 43	2 Way ANOVA	Tukey's
Sheet 44	Rotarod (Males)	2MO	14	15	Sheet 44	Mixed-Effects Model (REML)	Tukey's
Sheet 45	Rotarod (Females)	2MO	15	15	Sheet 45	Mixed-Effects Model (REML)	Tukey's
Sheet 46	Rotarod (All)	2MO	29	30	Sheet 46	Mixed-Effects Model (REML)	Tukey's
Sheet 47	Rotarod (Males)	9MO	14	15	Sheet 47	2 Way ANOVA	Dunnett's
Sheet 48	Rotarod (Females)	9MO	14	14	Sheet 48	2 Way ANOVA	Dunnett's
Sheet 49	Rotarod (All)	9MO	29	30	Sheet 49	2 Way ANOVA	Dunnett's
Sheet 50	Rotarod (Males)	16MO	12	12	Sheet 50	Mixed-Effects Model (REML)	Tukey's
Sheet 51	Rotarod (Females)	16MO	12	12	Sheet 51	Mixed-Effects Model (REML)	Tukey's
Sheet 52	Rotarod (All)	16MO	24	24	Sheet 52	Mixed-Effects Model (REML)	Tukey's
Sheet 53	Tube Test (All, Males, and Females)	16MO			Sheet 53	O. vs E.	NA
Sheet 54	Hindlimb Clasping (Males)	3-65wk	14	15	Sheet 54	Mixed-Effects Model (REML)	NA
Sheet 55	Hindlimb Clasping (Females)	3-65wk	14	14	Sheet 55	Mixed-Effects Model (REML)	NA
Sheet 56	Weight (Males)	3-65wk	14	15	Sheet 56	Mixed-Effects Model (REML)	Tukey's
Sheet 57	Weight (Females)	3-65wk	14	14	Sheet 57	Mixed-Effects Model (REML)	Tukey's

Table S3: Patient Information

Patient Information for PLA Experiments

Patient ID	Source	Type	Sex	Age	Pathological Diagnosis	Region	TDP-43 Pathology
S02471	Harvard BioBank	Affected	M	43	ALS	Superior Frontal Gyrus (BA9/46)	Not stated
S02650	Harvard BioBank	Affected	M	68	ALS	Superior Frontal Gyrus (BA9/46)	Not stated
S04139	Harvard BioBank	Affected	F	69	ALS	Superior Frontal Gyrus (BA9/46)	Yes
S08526	Harvard BioBank	Affected	M	54	ALS	Superior Frontal Gyrus (BA9/46)	Not stated
A14-101	UofT	Affected	F	67	ALS/FTLD-TDP	Orbitofrontal cortex	Yes
A14-88	UofT	Affected	M	74	ALS/FTLD-TDP	Orbitofrontal cortex	Yes
A15-07	UofT	Affected	F	59	ALS/FTLD-TDP	Orbitofrontal cortex	Yes
NP23-02	CHEO/uOttawa	Unaffected Control	F	74	Normal pressure hydrocephalus	Dorsolateral PFC (middle frontal gyrus)	No
NP23-03	CHEO/uOttawa	Unaffected Control	F	15	Normal	Dorsolateral PFC (middle frontal gyrus)	No
NP23-08	CHEO/uOttawa	Unaffected Control	M	90	Lewy body disease	Dorsolateral PFC (middle frontal gyrus)	No
NP23-14	CHEO/uOttawa	Unaffected Control	M	63	Cerebral amyloid angiopathy, small lymphocytic vasculitis	Dorsolateral PFC (middle frontal gyrus)	No
NP23-01	CHEO/uOttawa	Unaffected Control	M	68	Microangiopathy, old occipital infarct	Dorsolateral PFC (middle frontal gyrus)	No
S00057	Harvard BioBank	Unaffected Control	M	68	Unaffected	Superior Frontal Gyrus (BA9/46)	No
S09955	Harvard BioBank	Unaffected Control	F	66	Unaffected	Superior Frontal Gyrus (BA9/46)	No
S10700	Harvard BioBank	Unaffected Control	M	52	Unaffected	Superior Frontal Gyrus (BA9/46)	No
S11777	Harvard BioBank	Unaffected Control	M	44	First Degree Relative Schizophrenia	Superior Frontal Gyrus (BA9/46)	No

Patient Information for Aging Study

Patient ID	Age (Yrs)	Sex	Race	PMI (Hr)	Additional Information
					Subject was left unattended for about 5 minutes and was found in a swimming pool.
					Neuropath report findings: Unremarkable infant brain. Developmentally appropriate for the stated age.
4455	1.15748	Female	White	39	Autopsy report findings: Heavy and wet lungs. Scalp with 1/4 inch superficial contusion of left forehead. No lethal trauma. No evidence of infectious infiltrates or malignancies.
5113	36.22205	Male	Black or African-American	20	Donor was a 36 year old black male with a history of LVM. His recently started taking Lipitor. No history of drug, tobacco or ETOH abuse.
5182	86.00787	Male	White	23	Donor was found unresponsive at the bottom of the steps of his home. He was taken to the ER and pronounced. Donor had a history of heart disease and HBP.
5235	28.36063	Male	White	24	Donor was found unresponsive by his girlfriend. The evening before donor was consuming ETOH and smoking marijuana. Donor was a non-smoker and was not currently taking any medications.
5352	81.12756	Male	White	17	Donor was the driver of a MVA. No medical history available.
5391	8.453543	Male	White	12	Donor was an 8 year-old male who drowned in a river. Donor does not have any significant medical history and was not taking any medications.
5511	80.13701	Female	White	10	Medical Examiners Opinion: This 84 year old, white male, died of Atherosclerotic Cardiovascular Disease. Chronic Obstructive Pulmonary Disease and renal failure are contributing medical conditions. Abrasions of the forehead are consistent with collapse. The manner of death is NATURAL. The deceased had not been consuming alcoholic beverages prior to death.
5541	84.08346	Male	White	12	Pathologic diagnoses states that the decedent had severe Coronary Artery Disease, aortic valve calcifications, Cardiomegaly, Biventricular Dilation, a history of Congestive Heart Failure and a Pacemaker Implantation as a result of having Atherosclerotic Cardiovascular Disease. Along with COPD, the decedent had Hepatic Cysts, Renal Retention Cysts, a medical history of Renal Failure and Abrasions of the forehead. Donor has a medical history of H1N1, seasonal allergies, and family history of arrhythmias.
5558	5.546457	Male	White	19	Pathologic Diagnoses concludes that due to the Anomalous Left Coronary Artery with Complications the subject had an anomalous left main coronary artery arising in right sinus with acute angle take-off and proximal course between aorta and pulmonary artery. Subject also had circumferential subendocardial necrosis with contraction bands and interstitial hemorrhage, left ventricle, consistent with reperfusion injury, diffuse pulmonary hemorrhage with focal acute bronchopneumonia, early hypoxic-ischemic encephalopathy, focal ischemic acute renal tubular necrosis, focal ischemic change of small intestines and status post extracorporeal membrane oxygenation. Medical Examiners Opinion: This 5 year old white male died of Anomalous Left Coronary Artery with Complications. The manner of death is NATURAL. Toxicological analyses were negative for alcohol and drugs.
5576	2.453543	Male	Black or African-American	25	

					PATHOLOGIC DIAGNOSES: Along with acute bronchopneumonia, the decedent had a history of chronic diarrhea due to chronic loperamide use. Lastly deceased had cardiomegaly (heart weight 460 grams) MEDICAL EXAMINER'S OPINION: This 32 year old white female died of acute bronchopneumonia. A significant contributing condition was chronic Loperamide use. She had a history of chronic diarrhea and abdominal pain. She had a high level of Loperamide in her blood and this drug can depress respiratory function; however, given her history of chronic diarrhea, it is possible that she took this medication chronically and was tolerant to its effects. The manner of death is natural. Subject found on floor of home bleeding from his nose, unresponsive and not breathing. PATHOLOGIC DIAGNOSES: Mild concentric left ventricular hypertrophy with focally thickened intramural coronary arteries. The heart also showed fragmentation of compact atrioventricular node with potential accessory conduction pathways. MEDICAL EXAMINERS OPINION: This 17 year old African American male died of cardiac arrhythmia due to concentric left ventricular hypertrophy. Fragmentation of conduction pathway is a contributory cause of death. The manner of death is natural. Postmortem toxicology testing for alcohol and drugs negative. PATHOLOGIC DIAGNOSES: Due to reactive airway disease the decedent had hyper-inflated lungs with thick mucus plugs in the upper and lower airways, as well as inflammatory infiltration of bronchial walls with many eosinophils. MEDICAL EXAMINERS OPINION: This 20 year old white female died of reactive airway disease consistent with Asthma. The manner of death is natural. Postmortem toxicological testing was negative for drugs and alcohol. PATHOLOGIC DIAGNOSES: The factors that led to the hypertensive atherosclerotic cardiovascular disease are cardiomegaly (700 grams), left ventricular hypertrophy, left ventricular and left atrial dilatation, moderate to focally severe calcific coronary atherosclerosis, intra-plaque hemorrhage of mid left anterior descending artery, remote transmural infarct of lateral left ventricle, nephrosclerosis, severe cerebrovascular atherosclerotic disease and remote cerebral infarct. MEDICAL EXAMINERS OPINION: This 82 year old caucasian male died of hypertensive atherosclerotic cardiovascular disease. The manner of death is natural. Neuropath report findings: - Cerebrovascular atherosclerosis, severe - Remote infarct, left inferior parietal lobule, 1.5 cm
5629	32.12283	Female	White	42	
5643	17.1937	Male	Black or African-American	14	
5646	20.26457	Female	White	23	
5657	82.1685	Male	White	22	

					The following definitions are used for infant disorder names for cases with this notice: 1. "Unaffected Control" - If there is clear positional asphyxia or other identified cause. 2. "Unaffected Control, Co-Sleeping; SUDI, Suspected" - Any case where there is someone else in bed, but no clear indication of asphyxia. 3. "Unaffected Control, Unsafe Sleeping Conditions; SUDI, Suspected" - In bed alone but in prone position or with unsafe bedding. 4. "SUDI" – Sudden Unexplained Death in Infancy. Does not meet the criteria for 2 or 3. No apparent explanation for death. SUDI is used in place of "SIDS" by some medical examiners; SUDI is assigned by medical examiner. 5. "SIDS" - In crib alone in supine position w/o excessive bedding; Assigned by medical examiner. Cause of Death assigned by ME = Viral Syndrome Manner of Death assigned by ME= Natural
5708	0.245669	Female	Unknown	31	PATHOLOGIC DIAGNOSES: Adenovirus was isolated in cultures and identified by monoclonal stasis (postmortem nasopharyngeal swab culture study). As a result of the viral syndrome, the subject had laryngitis, focal acute pneumonia and otitis media. MEDICAL EXAMINERS OPINION: This 5 month old hispanic female infant died of viral syndrome. The manner of death is natural. Toxicological analyses for alcohol and drugs were negative. Neuropath report findings: Autopsy report findings: The decedent died of Occlusive Pulmonary Thromboemboli. The obesity is a contributing factor to death. The manner of death is natural.
5751	25.15276	Female	Black or African-American	24	Neuropath report findings: -Unremarkable adult brain. Autopsy Findings: Subject died of Pulmonary Thromboembolism. Her past medical hx is significant for atherosclerotic cardiovascular disease (status post myocardial infarction and coronary artery bypass graft surgery) and diabetes mellitus. The manner of death is NATURAL. Postmortem toxicology testing for alcohol was negative, however postmortem toxicology for drugs was positive for promethazine.
5762	39.2315	Female	White	19	Toxicology Report: The subject tested negative for both Ethanol and a comprehensive other drugs test. The subject tested positive for Promethazine 0.05 mg/L. Subject was found hanging at residence, no signs of foul play, drugs, or ETOH. Family mentioned no hx or mention of suicide. Autopsy Findings: This 18-year-old female died of hanging. Investigation and autopsy findings indicate that the manner of death is suicide. The deceased had not been consuming alcoholic beverages prior to death.
5822	18.55748	Female	Asian	39	Toxicology Findings: the subject tested negative for both ethanol and a comprehensive drug test. Final NP Findings: -unremarkable adult brain and spinal cord. Decedent was reportedly very upset when he was not allowed to play football at his school due to a sports related injury. He wrote a 2 page suicide note and sent a text to a friend advising him what he was going to do. The NOK found him hanging from a basketball hoop bracket by a belt. Decedent had no known prior suicidal ideations or attempts.
5879	15.02362	Male	Black or African-American	18	Autopsy Findings: The 15-year-old subject died of asphyxia by hanging, based on the investigation and autopsy findings, the manner of death is classified as suicide. Toxicology Report of Findings: Decedent tested negative for Ethanol and other drugs. Neuropath report findings: No acute or traumatic lesions are identified. All sections were stained for Tau immunohistochemistry and were negative. Unremarkable brain.

					Neuropath report findings: No acute or traumatic lesions are identified. Unremarkable infant brain and spinal cord.
					Autopsy Report of Findings: This 12 month 28 day old infant died of Sudden Infant Death Syndrome (SIDS). Investigation revealed that the infant was placed on his back in a playpen by a caregiver and found unresponsive several minutes later. An autopsy revealed no evidence of injury or significant natural disease processes and police investigation found no suspicions of foul play. The manner of death is natural.
5991	1.044094	Male	White	24	Toxicology Report of Findings: The subject tested negative for ETOH and drugs.
					Subject with hx of asthma had an asthma attack at home. No hx of seizures, fall, or trauma. No suspicious circumstances.
5925	7.40315	Female	Black or African-American	30	Autopsy Findings: This 7-year-old African American female died of Asthma. The manner of death is natural. Postmortem toxicological analysis was negative for drugs and alcohol.
					Subject was found unresponsive in the bathroom @ work & could not be resuscitated. Subject has a hx of social drinking and marijuana use. Neuropath report findings: No acute or traumatic lesions are identified. Unremarkable adult brain.
5926	21.38268	Male	Black or African-American	27	Autopsy Findings: Subject is a 21-year-old black male with a hx of social drinking and marijuana use. Subject's family has a hx of heart disease and hypertension. Subject had recent complaints of not feeling well and chest pain, subject was found unresponsive in bathroom of grocery store where he worked, could not be resuscitated.
					Possible exacerbation of asthma. Subject was not feeling well earlier in day. Went to school, participated in gym class. Utilized inhaler twice in gym, because extremely SOB.
5936	14.38425	Female	Black or African-American	23	Autopsy Findings: This 14-year-old adolescent female died of status asthmaticus. Reportedly, the decedent suffered from asthma and had an acute asthma attack while in the school gym. The treatment could not reverse her condition. The manner of death is natural. Toxicology Report: Subject tested negative for drugs and alcohol.
					The following definitions are used for infant disorder names for cases with this notice: 1. "Unaffected Control" - If there is clear positional asphyxia or other identified cause. 2. "Unaffected Control, Co-Sleeping; SUDI, Suspected" - Any case where there is someone else in bed, but no clear indication of asphyxia. 3. "Unaffected Control, Unsafe Sleeping Conditions; SUDI, Suspected" - In bed alone but in prone position or with unsafe bedding. 4. "SUDI" - Sudden Unexplained Death in Infancy. Does not meet the criteria for 2 or 3. No apparent explanation for death. SUDI is used in place of "SIDS" by some medical examiners; SUDI is assigned by medical examiner. 5. "SIDS" - In crib alone in supine position w/o excessive bedding; Assigned by medical examiner.
5947	0.28189	Male	White	11	Cause of Death assigned by ME = Asphyxia Manner of Death assigned by ME = Accident
					Subject was found between the wall and the bed. Subject was born full term, no medical hx. Accident. Final Neuropathologic Diagnosis: unremarkable infant brain and spinal cord.
					Autopsy Findings: This 5 month 26-day-old white male died of asphyxia. Per investigation, he was discovered to be unresponsive, with his head wedged between the mattress and headboard of an adult bed. Scene photographs show faint impression of the left side of the head consistent with reported circumstances. Autopsy showed no injuries. Autopsy showed no significant injuries, and no anatomic abnormalities or disease processed. The manner of death is accident.
5977	6.390551	Female	White	22	Subject was involved in a house fire, taken to ER & pronounced. Subject has a hx of congenital diaphragmatic hernia-nissen fundification and gastric feeding tube, flu shot. Subject tested negative for HIV 1 AB test, HIV 2 Ab test, RPR Syphilis test, HCV Ab test, HBc Ab test, and HBs Ag Test.
					Final Neuropathologic Diagnosis: unremarkable brain.
6032	4.080315	Male	White	25	4 year old died of head and neck injuries sustained as a rear seat passenger of a vehicle which left the roadway striking a tree head-on. The manner of death is accident.
					Neuropath report: Subarachnoid hemorrhage, right lateral frontal lobe Subarachnoid hemorrhage, ambient cistern
13019	23	F	W	15.3	NONE

13152	23	M	B	18.5	CONCUSSION NOS, TWO, AGE 18, MILD; MIGRAINE WITHOUT AURA, TWICE MONTHLY
13301	17	M	B	17.8	HEAD INJURY, AGE 11, WITHOUT COGNITIVE SEQUELAE OR BEHAVIORAL/PERSONALITY CHANGES
13323	24	F	W	25.0	NONE
13237	25	M	W	22.3	NONE
13260	30	F	W	12.9	NONE
13291	37	M	B	18.0	NONE
13344	33	F	W	12.1	NONE
13392	26	M	W	20.8	HEPATITIS C
13033	59	F	W	5.9	DIABETIC NEUROPATHY; BELL'S Palsy, AGE 28 WITH MILD RECURRENCE AGE 53; CEREBROVASCULAR ACCIDENT, ISCHEMIC LEFT THALAMIC, AGE 58. SUBARACHNOID HEMORRHAGE CONSISTENT WITH BFT.
13085	57	M	W	22.4	NONE
13114	54	F	B	13.7	NONE
13134	53	M	W	20.1	NONE
13250	49	F	W	11.3	NONE
13258	42	M	W	14.6	NONE
13302	46	M	W	12.4	NONE
13312	50	F	W	28.4	AMYLOID ANGIOPATHY IN FRONTAL LOBE & HIPPOCAMPUS BUT NOT BRAINSTEM.
13330	41	F	W	7.4	MIGRAINES
13353	47	M	B	23.4	NONE
13052	66	M	W	27.3	CARPAL TUNNEL SYNDROME. MILD ALZHEIMER'S DISEASE-RELATED PATHOLOGY. NOT UNEXPECTED IN PERSONS OF THIS AGE.
13091	63	M	W	25.9	SUBSTANTIA NIGRA ONE NEURON TAU+
13107	61	M	W	16.7	NONE
13122	68	F	B	26.5	NONE
13167	64	M	W	23.9	TAU AGGREGATES IN HIPPOCAMPAL NEURONS ARE ATYPICAL. PRESENT PRIMARILY IN CA1 AND CA2 BUT DO NOT EXTEND INTO SUBICULUM, AND NONE ARE IN ENTORHINAL CORTEX. H&E APPEARANCE OF NEURONS IS EOSINOPHILIC GRUMOUS DEGENERATION RATHER THAN THE BLUE, FIBRILLARY FLAME-SHAPED TANGLES NORMALLY SEEN. ETIOLOGY POSSIBLY RELATED TO CARDIAC HISTORY.
13184	63	M	W	19.2	PERIPHERAL NEUROPATHY, AGE 63, SECONDARY TO PREVIOUS BACK SURGERY. SINGLE NEURON IN SUBSTANTIA NIGRA HAD A TANGLE. SEVERAL NEUROPIL THREADS.
13232	65	M	W	23.9	ALPHA-SYNUCLEIN STAINING WAS MINIMAL. IN THE NIGRA THERE WERE ONLY SCATTERED THREAD-LIKE PROCESSES WITHOUT ANY DISTORTION. A SINGLE NEURON IN THE ENTORHINAL CORTEX WAS POSITIVE, AND THERE WERE FAR FEWER LEWY NEURITES THAN SEEN IN NIGRA. MILD AND UNLIKELY TO BE CLINICALLY SIGNIFICANT.
13387	60	M	W	11.3	NONE
13394	65	F	W	16.8	MODERATE NUMBERS OF NEURITIC PLAQUES BUT NO NEUROFIBRILLARY TANGLES. LIKELY NO OR ONLY MILD CHANGES IN COGNITION, CLINICALLY.
13428	64	M	W	9.2	CEREBRAL ARTERIAL ANEURYSM FIRST DOCUMENTED AGE 40; OBSTRUCTIVE SLEEP APNEA

Table S4: Reagents

Due to size and complexity, table can be found online:

<https://www.biorxiv.org/content/10.1101/2024.04.12.589206v1.supplementary-material>

Chapter 3: Characterizing the differential distribution and targets of Sumo1 and Sumo2 in the mouse brain

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3.1 Statement of Author Contributions

M.W.C.R., M.T., N.B., and **T.R.S.** conceived study and designed experiments. M.W.C.R., M.T. and N.B., designed the knock-in mice. **T.R.S.** conducted experiments and analyzed data for protein and RNA analyses, mass spectrometry experiments, whole brain imaging analyses, and immunofluorescent experiments in primary neurons. **T.R.S.** and J-L.A.P. cultured and maintained primary cortical neurons. Z.A.F., H.M.G., and T.T.N., performed staining in mouse tissue samples for comparison regional and cellular expression between His₆-HA-SUMO1 and His₆-HA-SUMO2 mice. M.T. and M.M. performed immunofluorescence experiments in His₆-HA-SUMO1 and HA-SUMO2 mice, performed synaptic co-localization experiments, and subcellular fractionation experiments. T.T.N. performed Proximity Ligation Assays in primary neuron culture. M.M.H. and S.M.C. maintained mouse lines at uOttawa; M.T. maintained mousselines at Max Plank. **T.R.S.** and M.W.C.R. wrote the manuscript alongside M.T. and N.B. and all authors were involved in editing versions of the manuscripts.

**Characterizing the differential distribution and targets of
Sumo1 and Sumo2 in the mouse brain**

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KEYWORDS

SUMOylation, Sumo2, Mouse Model, Whole Brain Imaging

3.2 Abstract

SUMOylation is an evolutionarily conserved eukaryotic posttranslational protein modification with broad biological relevance. Differentiating between the major SUMO paralogs and uncovering paralog-specific functions *in vivo* has long been very difficult. To overcome this problem, we generated His₆-HA-Sumo2 and HA-Sumo2 knock-in mouse lines, expanding upon our existing His₆-HA-Sumo1 mouse line, to establish a 'toolbox' for Sumo1-Sumo2 comparisons *in vivo*. Leveraging the specificity of the HA epitope, we performed whole brain imaging and uncovered regional differences between Sumo1 and Sumo2 expression. At the subcellular level, Sumo2 was specifically detected in extra-nuclear compartments, including synapses. Immunoprecipitation coupled with mass spectrometry identified shared and specific neuronal targets of Sumo1 and Sumo2. Target validation using proximity ligation assays provided further insight into the subcellular distribution of neuronal Sumo2-conjugates. The mouse models and associated datasets provide a powerful framework to determine the native SUMO 'code' in cells of the central nervous system.

3.3 Introduction

Post translational modifications (PTMs) can alter the secondary, tertiary, and quaternary structure of proteins and thereby dynamically regulate their function. Although many PTMs involve the covalent conjugation of small moieties, such as phosphate, glycosyl, or acetyl groups, protein function is also modulated by reversible covalent conjugation of large molecules, including the protein Ubiquitin and Ubiquitin-like modifiers, such as NEDD8, or SUMOs¹⁻⁷.

Conjugation of SUMOs (“Sumo” in mice) through SUMOylation is a dynamic and essential process that has been conserved throughout eukaryote evolution(Celen and Sahin, 2020;

Ilic et al., 2022). In its course, immature SUMOs are first matured by Sentrin Specific Protease (SENP) cleavage at a C-terminal diglycine motif that is essential for conjugation(Xu and Au, 2005). Next, SUMOs are activated in an ATP dependent manner and conjugated to the E1 ligase heterodimer consisting of SUMO Activating Enzyme 1 (SAE1) and Ubiquitin-Like Modifier Activating Enzyme 2 (UBA2)(Gong et al., 1999). SUMOs are then transferred to the sole E2 ligase of the system, SUMO conjugating enzyme UBC9 (UBC9, encoded by *UBE2I*), which is essential for SUMOylation(Desterro et al., 1997; Gong et al., 1997; Johnson and Blobel, 1997; Lee et al., 1998; Saitoh et al., 1998; Seufert et al., 1995). SUMOylation occurs at lysine residues of target substrates, typically in SUMO consensus motifs (Ψ -K-x-D/E; Ψ , large hydrophobic), either solely via UBC9 or upon facilitation by E3 ligases(Rodriguez et al., 2001; Sampson et al., 2001). SENPs readily deSUMOylate substrates, thus recycling SUMOs for subsequent conjugation rounds and allowing for highly dynamic substrate regulation (Nayak and Müller, 2014).

There are five human SUMO paralogs (SUMO1-SUMO5), of which SUMO1-SUMO3 have been studied most extensively as they are ubiquitously expressed and found in most vertebrates, whereas SUMO4 and SUMO5 are specific to humans and exhibit tissue-specific expression(Bouchard et al., 2021; Citro and Chiocca, 2013). SUMO1 is ~50% homologous to SUMO2 and SUMO3, whereas SUMO2 and SUMO3 are 97% homologous and often referred to together as SUMO2/3(Bohren et al., 2004). While SUMO paralogs have multiple redundant roles and can mutually compensate paralog loss in several contexts, each paralog also plays unique roles, e.g. by localizing to different distinct subcellular regions and by targeting different substrates(Citro and Chiocca, 2013; Ilic et al., 2022). Additionally, SUMO2, but not SUMO1 or SUMO3, is the only essential paralog so that its knock-out in mice causes major developmental defects and embryonic lethality by E10.5(Wang et al., 2014). The essentiality of SUMO2 for proper

development may be in part due to higher levels of SUMO2 in comparison to its paralogs(Wang et al., 2014; S. Yu et al., 2020), but is likely also a result of a specific subset of SUMO2 substrates in cells. Thus, characterizing the specific roles of SUMO2 is crucial to uncover the key molecular and cellular processes that define SUMO biology and the mechanisms by which SUMOylation affects development, disease processes, such as neurodegeneration or the pathological consequences of ischemic stroke(Bernstock et al., 2018; Mendler et al., 2016; Talamillo et al., 2020; Vertegaal, 2022; Yang and Paschen, 2015).

Several key challenges have limited studies in SUMO biology: (i) Differentiating between SUMO paralogs using antibodies is very difficult or even impossible due to the high degree of homology(Alexander J. Garvin et al., 2022); (ii) the highly dynamic nature of SUMOylation requires consideration of biological context, e.g. tissue, cell type, or cell state, for capturing and characterizing SUMOylation events, particularly *in vivo*; (iii) many tools and model systems used to study SUMOylation are limited, particularly when they rely on overexpression approaches, where off-target SUMOylation is a major potential confound. Consequently, protein SUMOylation has chiefly been studied in highly proliferating cells, where SUMOylation is now well known to control multiple nuclear processes, such as DNA repair or transcriptional control(Raman et al., 2013; Vertegaal, 2022). In contrast, the function of SUMOylation in extranuclear compartments, post-mitotic cells, or complex tissues is far less well understood. The role of extranuclear SUMOylation in postmitotic neurons is a prime example in this regard, with major controversies surrounding evidence supporting(Colnaghi et al., 2019; Henley et al., 2018; Lee et al., 2014; Marcelli et al., 2017; Matsuzaki et al., 2015; Schorova and Martin, 2016) and opposing(Daniel et al., 2018, 2017; Tirard et al., 2012) a key role of SUMO1 conjugation in synaptic compartments.

Our strategy to overcome some of the technical limitations in studying SUMO biology has been to generate knock-in (KI) mouse models that express endogenously tagged Sumo, allowing for stringent approaches that leverage the specificity and affinity of epitope tags to identify Sumo-targets and characterize their subcellular localization, using wild-type littermates as negative controls and flanked by studies on Sumo knock-out (KO) mice. The first allele we generated – a His₆-HA-Sumo1 mouse – overcame what we viewed as technical limitations of previous studies on the role of Sumo1 in neurons, and surprisingly did not provide evidence for the localization of Sumo1 at synapses (Daniel et al., 2018, 2017). While the His₆-HA-Sumo1 model facilitates the study of Sumo1 *in vivo*, expanding the toolkit to other Sumo paralogs will provide powerful opportunities to uncover the complexities of Sumo-mediated regulation.

We have now expanded our mouse genetic tool-generation efforts in SUMO biology to promote further studies on the role of SUMOylation *in vivo* and in complex cellular systems such as neurons. Specifically, we generated and characterized in parallel two novel knock-in mouse models that express either an HA-Sumo2 or a His₆-HA-Sumo2 fusion protein from the endogenous *Sumo2* locus to characterize Sumo2 and its substrates *in vivo*. Analogous to our work with the corresponding His₆-HA-Sumo1 mouse (Daniel et al., 2018, 2017; Tirard et al., 2012), these knock-in mouse lines allow to directly compare the localization and conjugation patterns of Sumo1 and Sumo2, which we did for the central nervous system (CNS). We used whole brain imaging to map the relative abundance of Sumo1 and Sumo2 in the adult mouse brain and observed that both are broadly distributed in the CNS but exhibit defined regional differences. Importantly, we found that Sumo2, unlike Sumo1 (Daniel et al., 2018, 2017; Tirard et al., 2012), is present in both, nuclear and extra-nuclear compartments, the latter including neuronal synapses. Given this distinct subcellular distribution, we explored native Sumo1 and Sumo2 targets using anti-tag

immunoaffinity purification from knock-in mice and mass spectrometry. We identified genuine Sumo2-specific targets in the adult mouse brain, including a subset of non-nuclear proteins. Moreover, we observed dynamic changes between Sumo1- and Sumo2-selective substrates. Finally, we validated several hits using proximity ligations assays to detect protein-Sumo interactions in cultured cortical neurons. This approach did not only provide an additional mode of validating native Sumo2 interactors in wild-type neurons, but also yielded spatial information, again demonstrating both nuclear and cytoplasmic interactions between Sumo2 and its substrates. Together, the two knock-in mouse lines and our data provide an important resource that lays the foundation of a 'SUMO-code' as an additional layer of molecular complexity in brain function.

3.4 Results

Generation of (His₆-)HA-Sumo2 mouse lines.

The use of epitope tags provides a versatile way to streamline the study of proteins by taking advantage of the highly selective nature of antibodies raised against these tags. In parallel institutions, we generated two independent alleles to facilitate the study of Sumo2 *in vivo*. We took advantage of a CRISPR/Cas9 approach to knock-in a 6xHis-hemagglutinin (*His₆-HA*)-tag into the amino-terminus of *Sumo2* (*His₆-HA-Sumo2*) in C57BL/6J mice (Figure S1A) in one case, mirroring that of a previously generated mouse line to study Sumo1 *in vivo* (Tirard et al., 2012). Moreover, we also generated an HA-Sumo2 knock-in mouse that does not have the His₆ tag. We observed that knock-in of either *His₆-HA* or *HA* to *Sumo2* resulted in hydrocephaly and premature death in heterozygous mice, indicating a likely hypomorphic effect of the epitope tag on a C57BL/6J background. Interestingly, backcrossing the mice to an FVB/N background alleviated these effects, resulting in no overt differences between mutant and wild-type FVB/N (WT) littermates in either line. Nevertheless, given the importance of Sumo2 for life, and the robustness of the HA-tag for visualization and biochemistry, we performed the bulk of our analyses on heterozygous *His₆-HA-Sumo2* (and heterozygous *His₆-HA-Sumo1*; also crossed to an FVB/N background) knock-in mice.

His₆-HA-Sumo2 enables the comparison of endogenous Sumo2 levels in the CNS.

To determine whether the addition of the HA epitope tag affects native Sumo levels, we assessed the levels of Sumo2/3 conjugates by Western blot, comparing total levels of unconjugated Sumo (“Free Sumo”) and Sumo-conjugated proteins from mouse brain lysates. We observed no change

in the abundance of Sumo2/3 conjugates in the brains of adult His₆-HA-Sumo2 mice compared to His₆-HA-Sumo1 or WT mice (Figure S1B). As decreased levels of Sumo1 conjugates were previously observed in homozygotes His₆-HA-Sumo1 mouse brain (Daniel et al., 2017), we thus measured whether there were any changes in the abundance of native Sumo1 conjugates in heterozygous His₆-HA-Sumo1 and His₆-HA-Sumo2 mice (Wang et al., 2014). Again, no changes were observed between either Sumo knock-in line and their WT counterparts, indicating that the heterozygous His₆-HA-Sumo2 or His₆-HA-Sumo1 allele does not alter overall levels of Sumo conjugates in mouse brain (Figure S1C). Additionally, we did not observe a significant effect on the expression of *Sumo1*, *Sumo2*, nor *Sumo3* transcript levels in either of the His₆-HA-Sumo knock-in mice (Figure S1D). Thus, the novel His₆-HA-Sumo2 mouse model enable the study of Sumo2 at endogenous levels *in vivo* without changes to the Sumo equilibrium.

The uniformity of the HA-tag epitope between the His₆-HA-Sumo1 and His₆-HA-Sumo2 mice allows for a more direct comparison of endogenous Sumo1 and Sumo2 levels *in vivo*. We assessed the relative levels *Sumo* expression in the central nervous system, specifically the cortex (CTX), cerebellum (CBM), olfactory bulbs (OB), spinal cord (SC) and remainder of the brain (termed “striatum-thalamus-brainstem for simplicity [S-Th-B]”) from adult mice (Figure 1A). Real-time qPCR analysis of WT mice show that *Sumo2* is the Sumo paralog most abundantly expressed in all regions of the CNS (Figure S1E). Additionally, *Sumo2* expression varies significantly between CNS regions, whereas *Sumo1* and *Sumo3* exhibit more uniform expression (Figure 1B). Western blot analysis from CNS regional lysates of His₆-HA-Sumo1, His₆-HA-Sumo2, and WT mice shows that levels of conjugated and free His₆-HA-Sumo1 indeed do not significantly vary between CNS regions, whereas free His₆-HA-Sumo2 levels reflect the higher expression and variations observed at the RNA level (Figure 1C, S1F, and S1G). Additionally, the

OB is particularly enriched for high molecular weight Sumo2-conjugated proteins suggesting that Sumo2 may play unique regional roles throughout the CNS (Figure S1F).

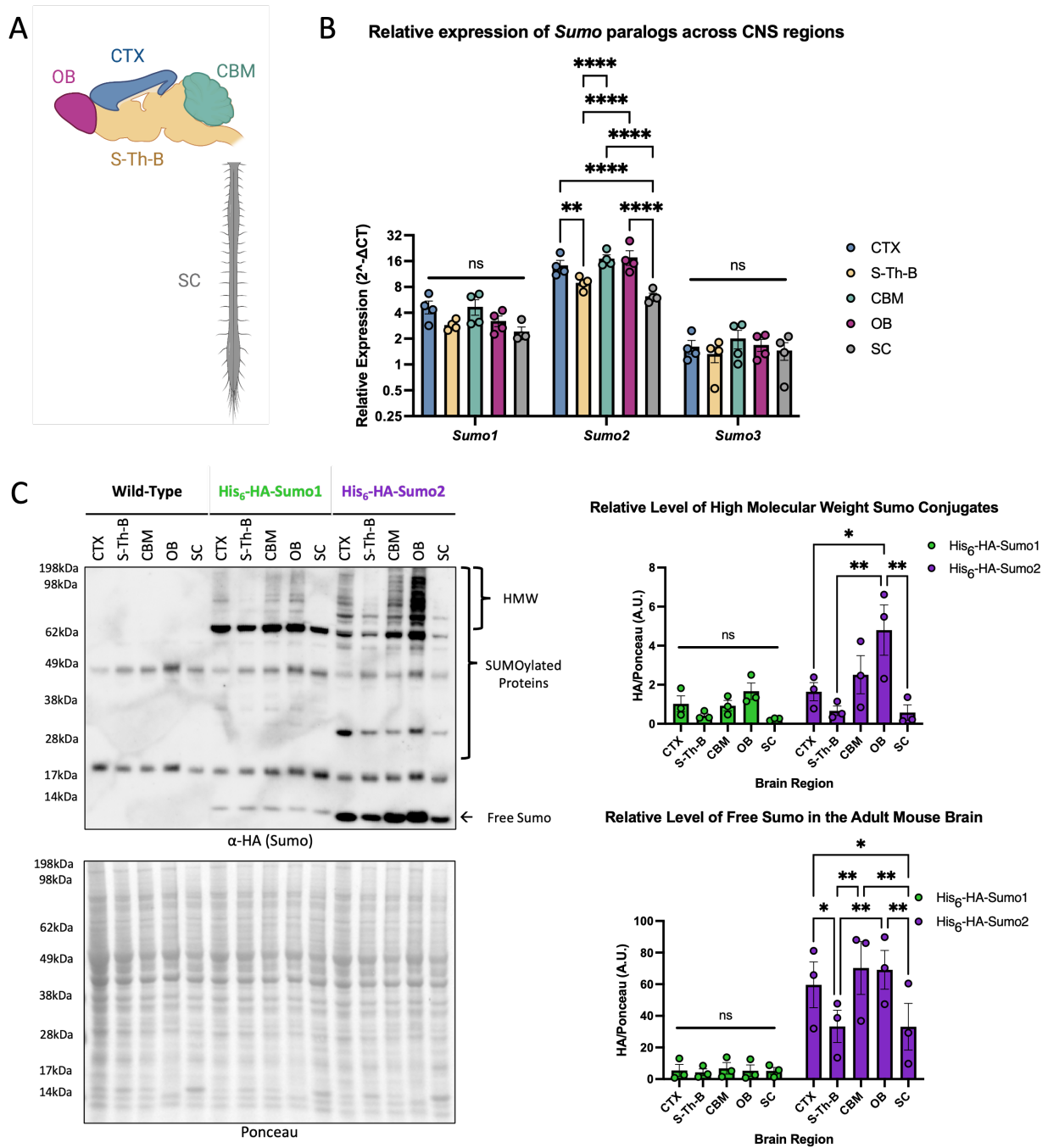


Figure 1: HA-epitope knock-in enables direct comparisons of differential levels of Sumo1 and Sumo2 and their conjugates in the murine central nervous system.

(A). Brain regions highlighted in the schematic were dissected for protein and RNA extraction and analyzed by Western blot using anti-HA antibody and RT-qPCR. (B). RT-qPCR analysis of WT mouse brain regions testing for significant regional changes for *Sumo1*, *Sumo2*, or *Sumo3* expression (N=4). (C). Western blot analysis of various regions of His₆-HA-Sumo1 and His₆-HA-Sumo2 mouse central nervous system. HA signal corresponding to free Sumo and SUMOylated proteins (HMW: High Molecular Weight) was normalized to ponceau and quantified via densitometry (N=3). Two-way ANOVA tests with Tukey's post-hoc multiple comparisons analysis were performed. Data are presented as mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. See also Figure S1.

Regional analysis of Sumo1 and Sumo2 throughout the mouse brain

As differences in Sumo levels are detectable from regional crude brain lysates, we leveraged the specificity of the HA-tag and performed whole brain clearing and mapping of Sumo1 and Sumo2 signals in the adult mouse brain. His₆-HA-Sumo1 and His₆-HA-Sumo2 mouse brains were cleared and immunolabeled against the HA-tag and NeuN before imaging using light sheet microscopy (Figure 2A, Video S1 and Video S2). Images were then analyzed and aligned to the Allen Brain Atlas to determine the relative levels of the His₆-HA-Sumo signals in defined brain regions (Table S1). It is worth noting that due to the different absolute abundance of Sumo1 (low) versus Sumo2 (high) in the brain, only qualitative Sumo1 vs Sumo2 comparisons were made. To generate relative maps of HA-Sumo distribution, the mean regional HA signal intensity was first normalized to the regional density of NeuN to account for changes in cell density before being averaged across hemibrains to account for tissue variability. Generally, Sumo1 and Sumo2 showed similar distributions throughout the mouse brain (Figure 2B-E), and we observed that both Sumo1 and Sumo2 were most abundant in cerebellar nuclei.

While Sumo levels were generally evenly distributed when characterizing gross anatomical regions, clear nuances in Sumo abundance were detectable at higher anatomical resolution. For example, in the hippocampus, Sumo levels were highest in the parasubiculum and presubiculum, and lowest in the *Fasciola cinerea* (Figure S2A & S2B). In the neocortex, we observed that Sumo levels were generally enriched in layers 2/3 (Figure 2B, 2D, & S2C), an area critical for integrative processing (Feldmeyer, 2012). Interestingly, while Sumo1 and Sumo2 often share similar distributions, we observed that Sumo2 is enriched in the anterior hypothalamus (Figure S2D), particularly in regions involved in the circadian rhythm, including the suprachiasmatic nucleus and subparaventricular zone (Drunen and Eckel-Mahan, 2021).

We investigated whether the brain-wide imaging findings could be validated by an orthogonal immunofluorescence assay (Figure S3). Looking at multiple brain regions, we found that Sumo1 and Sumo2 are broadly distributed through the somatosensory cortex. Interestingly, Sumo1 maintains a strictly nuclear localization, whereas Sumo2 is observed throughout the nucleus and in the cytoplasm and axons of cortical neurons. In the cerebellum, we found that Sumo2 is specifically enriched in granule cells, contrasting the strong Purkinje cell staining observed in His₆-HA-Sumo1 mice (Tirard et al., 2012). The hippocampus also displays characteristic nuclear staining for His₆-HA-Sumo1 in both the CA1 and CA3, whereas Sumo2 is seen throughout the nucleus and in the somata, and axons of hippocampal neurons. Thus, while Sumo1 and Sumo2 may share regional expression in the brain, the distinct localizations of Sumo1 and Sumo2 *in vivo* indicate that different targets of SUMOylation or different metabolism or conjugation dynamics of free Sumo likely play unique roles in neuronal regulation.

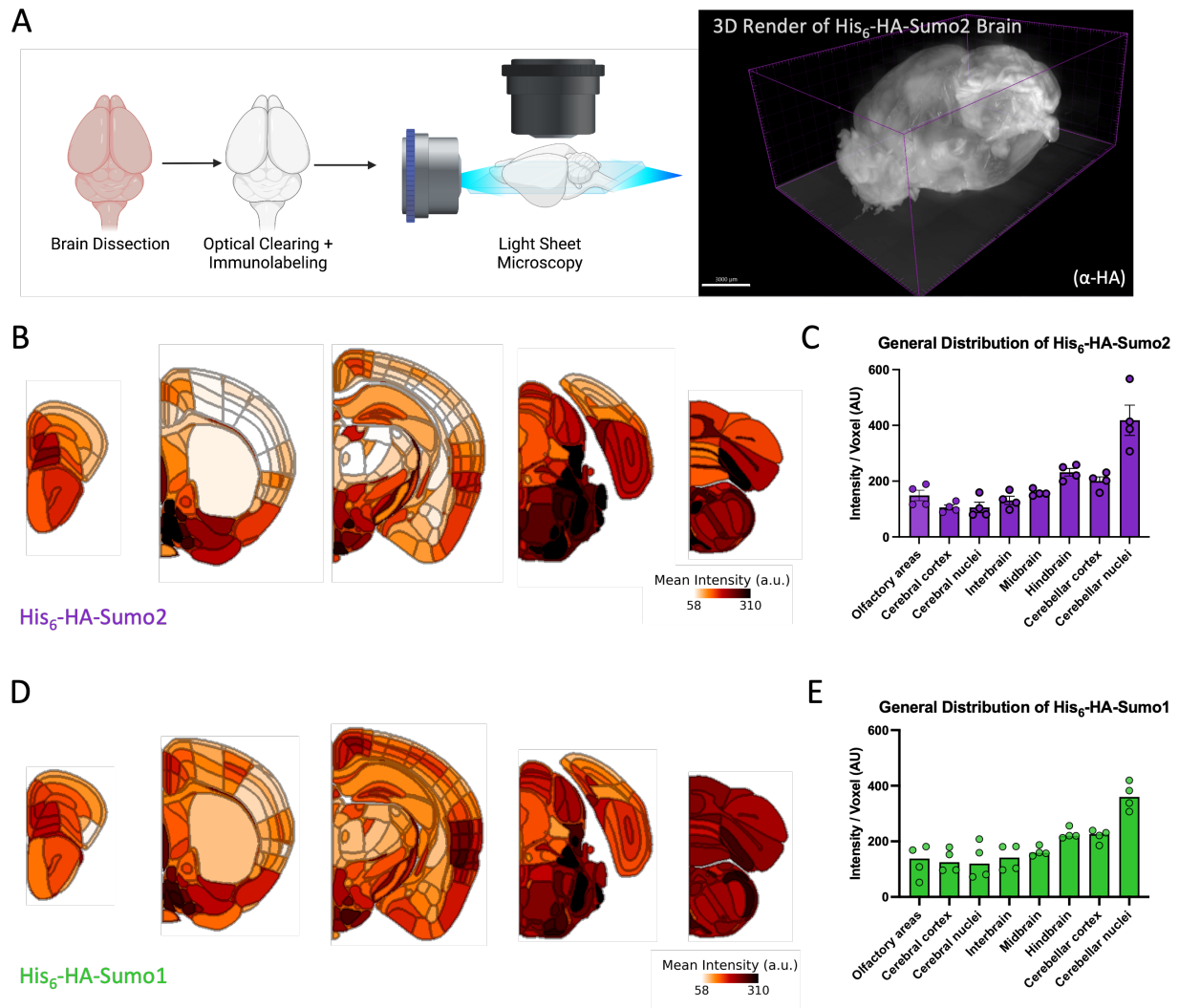


Figure 2: Whole brain imaging reveals the topographical distribution of Sumo paralogs.

(A). Schematic of brain clearing and light sheet microscopy for whole brain imaging with a 3D Imaris render of a His₆-HA-Sumo2 brain. **(B).** Heatmaps of anti-HA immunosignal intensity normalized to regional density quantified by NeuN staining and alignment to the Allen Brain Atlas across the His₆-HA-Sumo2 depicted brain region, averaged between hemibrains and biological replicates (N=2, two hemispheres/replicate). **(C).** Bar plot depicting the relative levels of anti-HA immunosignal per voxel averaged across across His₆-HA-Sumo2 brain regions defined by the Allen Brain Atlas. **(D).** Heatmaps of anti-HA immunosignal intensity normalized to regional density quantified by NeuN staining and alignment to the Allen Brain Atlas across the His₆-HA-Sumo1 depicted brain region, averaged between hemibrains and biological replicates (N=2, two hemispheres/replicate). **(E).** Bar plot depicting the relative levels of anti-HA immunosignal intensity normalized to regional density across His₆-HA-Sumo1 brain regions defined by the Allen Brain Atlas. Each datapoint in (C) and (D) is from the mean intensity from a single hemisphere (N=2, two hemispheres/replicate). Data are presented as mean $\pm$ SEM. See also Figure S2, Figure S3, Table S1, Video S1, and Video S2.

HA-Sumo2 localizes to synapses in vivo.

Consistent with our previous work, specific Sumo1 immunosignal was mainly observed in neuronal nuclei (Figure S3)(Tirard et al., 2012). However, we noticed that Sumo2 signal can also be found outside the nuclear compartment (Figure S3). Thus, we further characterized extra-nuclear Sumo2 signals using homozygous HA-Sumo2 mice. As in the His₆-HA-Sumo2 model, levels of Sumo2 conjugates were unaltered in total brain lysates from homozygous HA-Sumo2 mice as compared to WT littermates, ruling out an effect of the HA-tag on overall Sumo2 expression and conjugation levels (Figure S4A)(Tirard et al., 2012). Western blot analysis of brain subcellular fractions using an anti-HA antibody highlighted not only the difference in global levels of Sumo1 and Sumo2 conjugates, but also revealed that Sumo2 conjugates are enriched in non-nuclear fractions compared to their Sumo1 counterpart, including synaptic cytosol fractions S2 and S3 (Figure S4B & S4C). A weak but specific signal was also observed in crude synaptosomes (P2) and other synaptic membrane fractions, e.g. P3 and LP2, while the synaptic plasma membrane fraction (SPM) primarily appears devoid of Sumo2 conjugates (Figure S4B – S4C).

Cellular fractionation experiments are often burdened with cross contamination between fractions that can limit interpretations to assessing relative enrichment in each fraction. Well controlled immunofluorescent studies can overcome this limitation and provide spatial evidence for protein localization. As such, we used homozygous HA-Sumo2 mice to further characterize extra-nuclear Sumo2 by performing anti-HA immunolabeling of the hippocampal CA3 area, a region enriched in synapses (Figure S3). Brain sections from WT and HA-Sumo2 mice were immunolabeled for HA and neuronal markers for dendrites and synaptic compartments, and anti-HA immunosignals were quantified in HA-Sumo2 mice as compared to WT (Figure 3). Using confocal microscopy, we confirmed the presence of Sumo2 in extra-nuclear compartments, specifically along dendrites

and in synapses (Figure 3A). Quantification of HA-Sumo2 immunosignals in DAPI positive area confirmed the strong enrichment of Sumo2 conjugates in neuronal nuclei, in agreement with the well-known role of SUMOs as nuclear regulators (Figure 3A and B)(Hendriks and Vertegaal, 2016, 2015). In addition, HA-Sumo2 immunosignal was also observed in MAP2-positive regions and, strikingly, in synapses positives for Synapsin1, Shank2, Gephyrin, and/or vGlut1 (Figure 3C and D). Higher magnification images confirmed the presence of Sumo2 along MAP2 dendrites and at synapses (Figure 4 & S5). Quantification of the 3D object-based co-localization between the anti-HA immunosignal with the various synaptic markers confirmed the presence of Sumo2 at synapses, with HA-Sumo2 signal being significantly co-localized with Shank2, Synapsin 1, vGlut1 and vGAT (Figure 4C & S5, which shows a typical 3D reconstruction generated in Imaris). Co-localization of Sumo2 with Gephyrin was not obvious, most likely due to difficulties in immunolabeling inhibitory synapses with the method optimized for HA visualization. Altogether, our biochemical fractionation and immunofluorescence support the presence of Sumo2 at synapses.

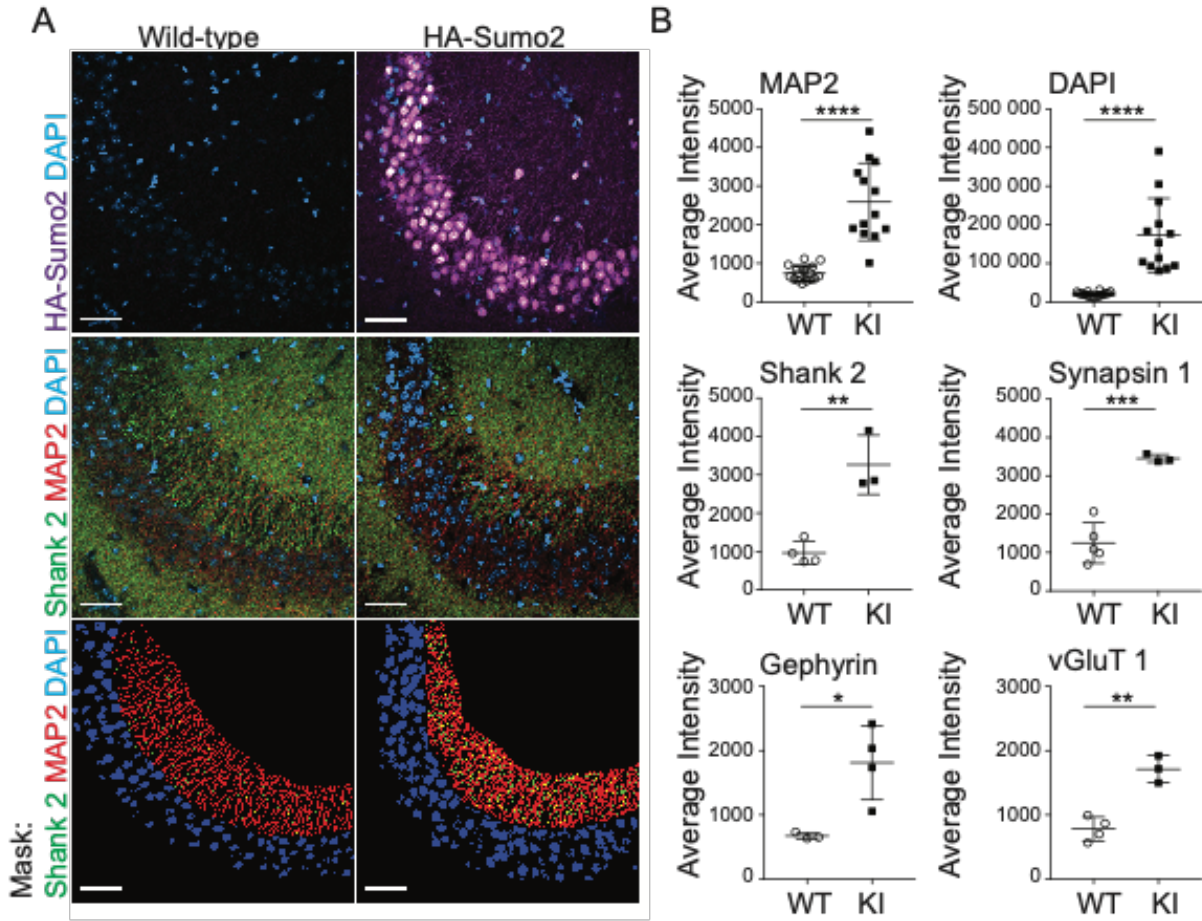


Figure 3: Extra-nuclear localization of Sumo2 in neurons of the hippocampal CA3 region.

(A). Anti-HA (purple), DAPI (blue), MAP2 (red), and anti-Shank2 (green) immunostaining of wild-type (left) and HA-Sumo2 KI (right) hippocampal CA3 region. Scale bar: 50 μ m. (B) Anti-HA signal in masked regions for each marker (exemplified for Shank2 in the bottom panel A) was quantified in both wild-type (WT) and HA-Sumo2 KI, and average intensity was calculated. N=3, * p <0.05, ** p <0.005, *** p <0.0005, **** p <0.0001. See also Figure S4.

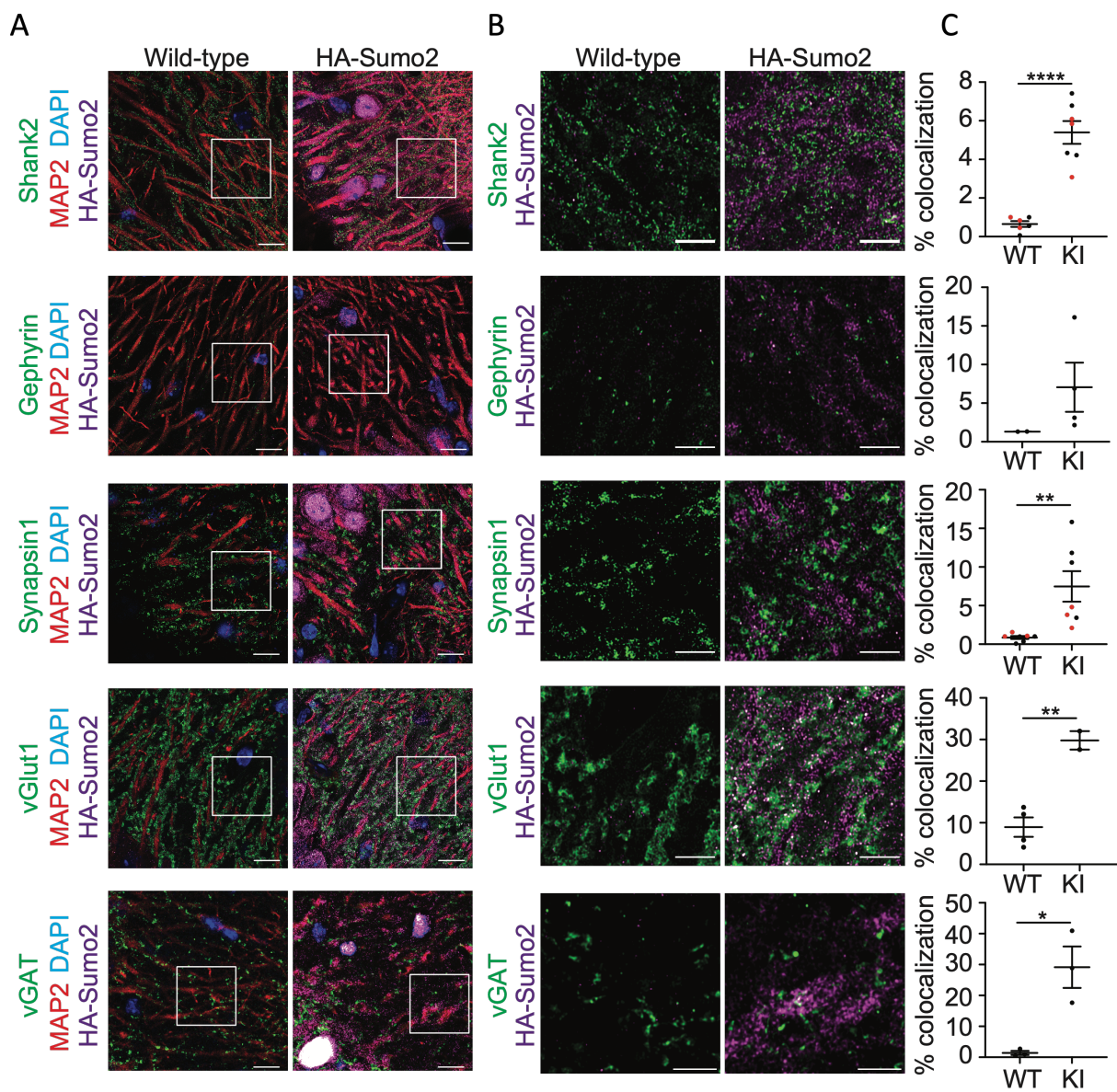


Figure 4: Sumo2 localizes at synapses *in vivo*.

(A). High magnification images of wild-type (left) and HA-Sumo2 KI (right) hippocampal CA3 region labelled with anti-HA (purple), DAPI (blue), MAP2 (red), and synaptic markers (green) Shank2, Gephyrin, Synapsin1, vGlut1 and vGAT1. Scale bar, 10 μ m. **(B).** High magnification images with equally scaled anti-HA intensity corresponding to the insets (white boxes) in A showing anti-HA (purple) immunostaining of wild-type (left) and HA-Sumo2 KI (right) hippocampal CA3 region immunolabelled with the synaptic markers (green) Shank2, Gephyrin, Synapsin1, vGLUT1 and vGAT1. Scale bar: 5 μ m. N=3. **(C).** The percentage of co-localization between each synaptic marker and the anti-HA signal was quantified in both wild-type (WT) and HA-Sumo2 KI. N=3. Data are presented as mean $\pm$ SEM. * p <0.05, ** p <0.005, **** p <0,0001. See also Figure S5.

HA-Sumo mice reveal convergent and contrasting neuronal Sumo1 and Sumo2 substrates.

The observation that there are regional and subcellular differences between Sumo1 and Sumo2 indicate divergent roles for the Sumo paralogs in the mouse brain. To uncover such molecular differences between Sumo paralogs, we performed HA-tag immunoprecipitation, under denaturing conditions to break apart standard protein-protein interactions, from whole brain lysate of WT, His₆-HA-Sumo1, and His₆-HA-Sumo2 mice, followed by mass spectrometric analysis to identify candidate targets of Sumo1 and Sumo2 *in vivo* (Figure 5A & S6A; Table S2). To rank proteins identified from the mass spectrometry dataset, we summated the total peptides from 4 replicates per genotype and then filtered proteins with >2-fold enrichment over proteins identified in the WT condition. Next, to determine high-stringency candidates, proteins were further filtered based on peptide counts being identified in at least 2/4 replicates in the HA-Sumo conditions, and no more than 1/4 replicate in the respective WT condition. Using these criteria, we identified 131 proteins enriched in the HA-Sumo1 and 75 proteins enriched in the HA-Sumo2 immunoprecipitations (Figure 5B & S6B; Table S2). Gene ontology and Reactome terms related to protein SUMOylation process (Figure 5B) were significantly enriched in both datasets (Figure 5C; Figure S6C; Table S2). Indeed, we identified critical components of the SUMOylation pathway including E1 SUMO ligases Sae1 and Uba2, the E2 SUMO ligase Ube2i, and E3 ligases Ranbp2(Pichler et al., 2002), Trim28(Liang et al., 2011), and Pml(Chu and Yang, 2011), validating the use of our mouse model to identify molecular substrates involved with SUMOylation.

To determine whether we could identify specific interactors of Sumo2, we compared the relative fold change of peptides identified in the His₆-HA-Sumo2 immunoprecipitation to that of the His₆-HA-Sumo1 immunoprecipitation and found that there are shared, specific, and

preferential interactors of Sumo2 vs. Sumo1 (Figure 5B, S6B & S6D; Table S2). We selected several hits from our mass spectrometry screen for validation via Western blot (Figure 5D). Ranbp2 and Rangap1 are consistently identified amongst the most abundantly SUMOylated proteins (Gareau et al., 2012; Geiss-Friedlander and Melchior, 2007; Saitoh et al., 1998) and were indeed validated to be shared targets of Sumo1 and Sumo2 in the mouse brain. Additionally, both proteins displayed a preference for Sumo1 modification in the mouse brain as previously described (Gareau et al., 2012). Conversely, the E3 ligase and transcriptional repressor Trim28 displayed a preference for Sumo2 modification in the adult mouse brain. Interestingly, the transcription factor Prox1, involved in neurogenesis and a marker of granule cells in the dentate gyrus and cerebellum (Lavado et al., 2010), was one of the most abundantly SUMOylated proteins targeted specifically by Sumo2 in our screen. We further tested the sensitivity of the model to detect more modestly SUMOylated proteins targeted by Sumo2 in the mouse brain, as measured by relative abundance in our immunoprecipitation coupled with mass spectrometry experiment. We confirmed that both Histone Deacetylase 2 (Hdac2) and General Transcription Factor 2i (Gtf2i, also known as Tf2-i) were Sumo2-modified (Figure 5D). Because of the dynamic nature of SUMOylation and the fact that Sumo only targets a fraction of the total pool of most substrates, biologically meaningful interactions may be buried under strong, constitutively SUMOylated targets in our screen. As a proof of concept, we picked a hit with low enrichment in the His₆-HA-Sumo2 immunoprecipitation by expanding the filtering criteria to a >1.5 fold enrichment over WT: Matrin3 (Matr3). We found that Matr3 was selectively SUMOylated in the His₆-HA-Sumo2 pulldown (Figure 5D), indicating that even at milder cut-off thresholds, SUMOylated substrates identified by mass spectrometry can be validated in an orthogonal assay. Taken together, these results show that the His₆-HA-Sumo2 mouse model is effective to identify SUMOylated proteins,

including proteins SUMOylated at relatively low levels *in vivo*, and can be used for targeted studies of protein substrates.

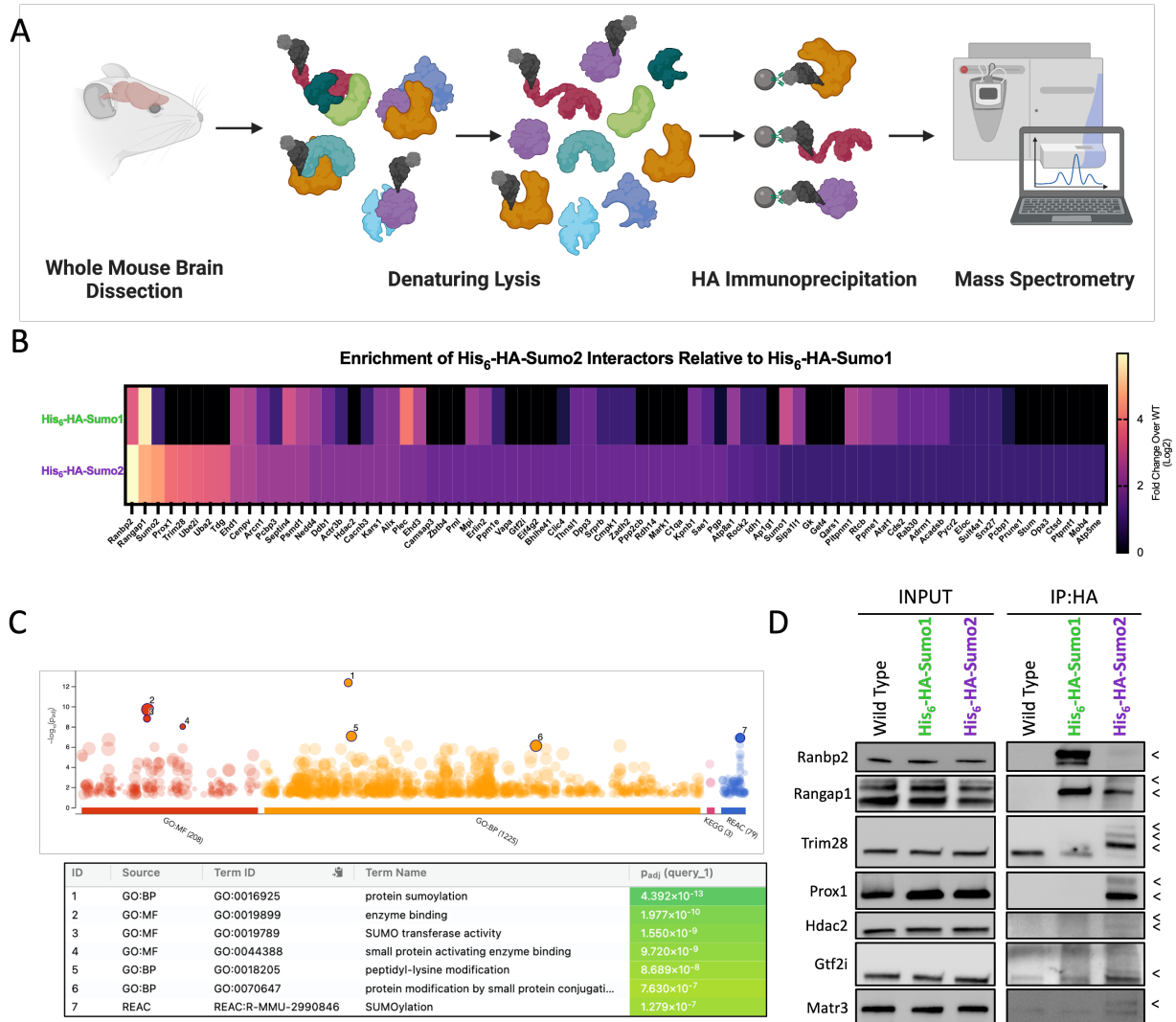


Figure 5: Neuronal Sumo2 has shared and distinct substrates compared to Sumo1 *in vivo*.

(A). Schematic of approach to identify His₆-HA-Sumo interactors from the adult mouse brain via anti-HA affinity immunoprecipitation and mass spectrometry (N=4). (B). Heat map depicting the relative peptide abundance of His₆-HA-Sumo2 interactors relative to levels in His₆-HA-Sumo1 immunoprecipitation. (C). gProfiler2 Gene Ontology analysis for His₆-HA-Sumo2 interactors. (D). Anti-HA affinity Immunoprecipitation followed by Western Blot analysis of His₆-HA-Sumo1 and His₆-HA-Sumo2 interactors as listed on the left side (N=3-6). Black arrowheads indicate protein SUMOylation inferred by shift in molecular weight. See also Figure S6 and Table S2.

Subcellular localization of Sumo interactions in neurons

Sumo1 and Sumo2 predominantly reside in the nucleus, and unsurprisingly, many of the interactors identified here and in other studies of protein SUMOylation are nuclear proteins. However, previous studies hinted at roles for SUMOylation outside of the nucleus (Hasegawa et al., 2014; Ilic et al., 2022; Wang et al., 2014; Watanabe et al., 2008; Yang and Paschen, 2015). During the validation of whole brain imaging, we observed that His₆-HA-Sumo2, while extensively localized throughout the nucleus, could also be observed within the somata and synapses of neurons *in vivo*, contrasting the predominantly nuclear membrane staining of His₆-HA-Sumo1 (Figure S3; Figure 3). The cytoplasmic localization of His₆-HA-Sumo2 in neurons indicates unique extranuclear roles *in vivo*. Indeed, we identified several predominantly extranuclear proteins via mass spectrometry in the anti-HA affinity purification from the His₆-HA-Sumo2 mice. To better visualize the subcellular localization of Sumo, we cultured primary cortical neurons from heterozygous His₆-HA-Sumo1, His₆-HA-Sumo2, and WT animals and co-stained them for HA and Map2 as a cytoplasmic marker. As previously observed, Sumo2 is predominantly distributed uniformly throughout the nucleus except for heterochromatic foci but is also observed at low levels in the cytoplasm (Figure S7A), contrasting with the anti-HA immunosignal observed for Sumo1, which predominantly localizes at the nuclear membrane. This indicates that Sumo2 specifically may play a larger role in regulating proteins outside of the nucleus.

To better assess the subcellular localization of neuronal SUMOylated proteins targeted by Sumo2, and to provide an additional level of validation in an orthogonal system, we used proximity ligations assays (PLA) against endogenous Sumo2/3 and targets of Sumo2 identified from our mass spectrometry screen to visualize interactions within WT primary cortical neurons (Figure 6A) (Ristic et al., 2016; Sahin et al., 2016). In this assay, proteins colocalizing within 40 nm will

produce a PLA signal suggesting a potential interaction. We selected proteins expected to interact with Sumo2/3 in the nucleus, Gtf2i and Matr3, as well as expected cytoplasmic interactors Rangap1, Cttd, Alix, and Kars1 for additional validation (Figure 6B & C). Gtf2i is distributed almost exclusively throughout the nucleus and interactions between Sumo2/3 and Gtf2i, inferred by the presence of PLA foci, are seen throughout the nucleoplasm. Interestingly, PLA foci were often observed to cluster around constitutive heterochromatin (Figure 6C, inset), indicating a potential role for Sumo2 in regulating Gtf2i at the periphery of heterochromatin in neurons. Matr3 is also localized throughout the nucleus and to a lesser extent in the cytoplasm, consistent with its identification in cytoplasmic processing bodies (Rajgor et al., 2016). Interestingly, PLA signals for Matr3 and Sumo2/3 are robust, indicating extensive interactions between the two proteins. Immunofluorescence staining of Rangap1, Cttd, Alix, and Kars1 demonstrate these proteins predominantly reside outside of the nucleus (Figure 6C). PLA between Rangap1, Cttd, and Kars1 with Sumo2/3 display an increase in the proportion of cytoplasmic foci relative to nuclear foci (Figure 6B), in addition to a substantial decrease in total nuclear PLA foci compared to nuclear localized proteins Gtf2i and Matr3 (Figure S7B). Importantly, for all PLA assays conducted, negative controls did not elicit a PLA signal (Figure S7C). Taken together, our PLA analyses of WT neurons provide an additional layer of validation of Sumo2-substrate interactions. With the added benefit of providing spatial resolution to the interactions, PLA data support a role for neuronal Sumo2 outside the nucleus.

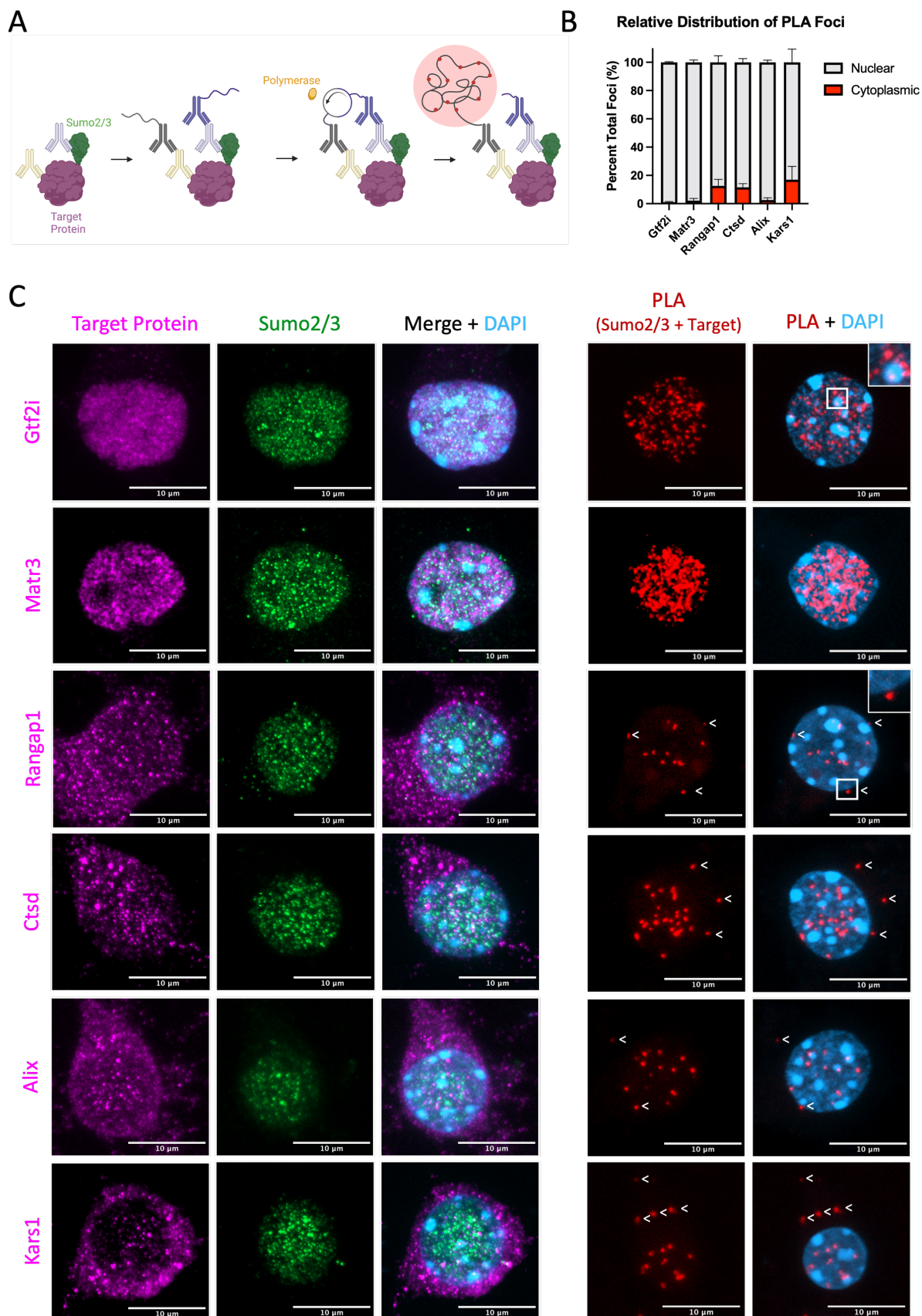


Figure 6: Established and newly identified Sumo2 substrates are present both in nuclear and extranuclear compartments in neurons.

(A). Schematic of the proximity ligation assay (PLA) strategy for native Sumo2/3 and target proteins in WT primary cortical neurons. (B). The relative proportion of PLA foci between the selected target proteins and Sumo2/3 was quantified and normalized within the nucleus (grey) versus outside of the nucleus (red) (N=3). (C). Representative Z-projected immunofluorescent images and PLA assays for selected target proteins identified from the mass spectrometry screen (N=3). White arrowheads indicate cytoplasmic PLA foci. Scale bar: 10 μ m. Data are presented as mean $\pm$ SEM. See also Figure S7.

3.5 Discussion

A constantly growing body of evidence demonstrates that SUMOylation plays key roles in the brain including neuronal development or learning and memory(Chen et al., 2014; D. Huang et al., 2020; Ripamonti et al., 2020; Talamillo et al., 2020; Wang et al., 2014; S. Yu et al., 2020). Furthermore, SUMOylation is linked to various brain disorders, including stroke, autism spectrum disorder, epilepsy, schizophrenia, or neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis^{61–68}. However, despite a clear connection to neuronal function, SUMOylation in neurons has remained somewhat enigmatic, mainly due to technical challenges and limitations to study this PTM(Daniel et al., 2017). We generated His₆-HA-Sumo2 and HA-Sumo2 knock-in mouse lines to overcome these technical limitations and to provide insight into the role of native SUMOylation *in vivo*. These mouse lines complement the previously generated His₆-HA-Sumo1 line(Tirard et al., 2012), enabling the direct comparison of Sumo1 and Sumo2 biology *in vivo* and the elucidation of specific Sumo2 functions.

We observed that *Sumo2* is more abundantly expressed throughout the CNS than *Sumo1* and *Sumo3* (Figure 1B). Interestingly, on both the RNA and protein levels, Sumo2 also displayed significant variations between CNS regions whereas Sumo1 levels did not significantly change. This may suggest that Sumo2 plays regional roles in the CNS. Additionally, we observed that the pool of free Sumo2 is significantly greater than that of Sumo1 in all CNS regions, and levels of free Sumo2 significantly differ between regions (Figure 1C). As Sumo2 has been described to respond to stimuli, such as stressors, the pool of free Sumo2 may reflect the dynamic nature of SUMOylation in each region with cells requiring more Sumo2 to be readily available to respond dynamically in the cellular environment (Hendriks et al., 2018). Future studies focusing on SUMO

dynamics in neuronal populations may help to uncover paralog and cell-type specific roles for SUMO-mediated regulation in the CNS.

Using whole brain imaging upon HA epitope labeling in His₆-HA-Sumo1 and His₆-HA-Sumo2 mice, we found that Sumo1 and Sumo2 are broadly distributed throughout the mouse brain. More detailed anatomical analyses revealed distinct differences between Sumo1 and Sumo2 levels across brain structures and some divergence between Sumo1 and Sumo2 levels in the brain. Levels of His₆-HA-Sumo1 are generally similar between cortical layers, whereas His₆-HA-Sumo2 levels are higher in layers 2/3 (Figure S2D). In the hypothalamus, we found that Sumo levels vary across anatomical regions (Figure S2E). Interestingly, regions involved in circadian rhythm control, including the subparaventricular zone (SBPV) and suprachiasmatic nucleus (SCH), show high levels of Sumo2. These findings are consistent with previous reports indicating that Sumo1 and Sumo2 differentially affect circadian-clock-related proteins, such as PER2 and CLOCK, and support the notion of a role of SUMOylation in regulating circadian rhythms (Chen et al., 2021; Drunen and Eckel-Mahan, 2021; Lee et al., 2015).

Importantly, we observed striking differences between the subcellular distribution patterns of Sumo1 and Sumo2. Western blot analysis of brain subregional fractions showed that Sumo2 is more abundant than Sumo1 (Figure 1C) and that Sumo2 conjugates, unlike Sumo1 conjugates, are prominently present in extranuclear fractions, including synaptic fractions (Figures S4B and S4C). However, brain subcellular fractionation is often unclear and nuclear material frequently leaks to non-nuclear fractions. Thus, immunofluorescence analysis is more powerful in determining the precise subcellular localization of Sumo conjugated proteins. More specifically, anti-HA immunolabeling of brain material from HA-Sumo2 mouse brains and WT littermates as negative controls also demonstrated the presence of extranuclear free Sumo2 and Sumo2 conjugates

(Figures 3, 4, and S5). Quantitative analyses of anti-HA immunosignals confirmed a partial co-localization of Sumo2 with pre- and post-synaptic markers at excitatory and inhibitory synapses (Figures 3A-B and 4A-C). These data, which were stringently controlled using WT material as negative control, partly align with a recent report describing Sumo2 localization at synapses (Colnaghi et al., 2019). On aggregate, our approach to study Sumo2 expression using the HA-Sumo2 mice allows for the specific assessment of Sumo2 immunosignals in neuronal sub-compartments and provides unequivocal evidence for the presence of Sumo2 at mammalian brain synapses. Thus, not only do Sumo1 and Sumo2 display differential expression and protein levels throughout the brain but are also localized and operational in different neuronal sub compartments. However, the functions of Sumo2 at synapses remain elusive. Importantly, there may be both direct (by Sumo-conjugation or -binding) and indirect roles for Sumo1 and Sumo2 in synaptic function that will have to be carefully assessed in the future to better understand how Sumo regulates synapse biology. In sum, the specificity provided by the His₆-HA-Sumo1 and (His₆-)HA-Sumo2 models enable direct comparisons between paralogs by overcoming key technical limitations. This will help to more accurately uncover specific roles for SUMOylation *in vivo*.

Sumo2 is the only functionally essential Sumo paralog, and, as mentioned, has key roles in neurodevelopment, nerve cell function, and neurological diseases (Drisaldi et al., 2015; Krumova and Weishaupt, 2013; Ripamonti et al., 2020; Stankova et al., 2018; Tai et al., 2016; Wang et al., 2014). To uncover some of the unique clientele targeted by Sumo2 conjugation, we performed HA-tag immunoprecipitation paired with mass spectrometry. As SUMOylation results in the covalent interaction between Sumo and the target substrate, mouse brains were lysed under denaturing conditions to promote the dissociation of non-covalent protein complexes prior to immunoprecipitation to help identify bona fide SUMOylation substrates. Using stringent criteria,

75 proteins were identified to be conjugated to Sumo2, ~50% of which were selective to Sumo2. Conversely, using the same criteria, we identified 131 candidate Sumo1 substrates, 98 of which were selective to Sumo1. “Canonical” synaptic proteins were not amongst the hit lists in these experiments, which is likely related to the fact that experiments were performed in whole adult brains lysates, where levels of SUMOylated synaptic proteins may be too low due to dilution effects. The dynamics of SUMOylation may add additional complications in capturing synaptic SUMOylation events as physiological conditions may differentially affect when synaptic proteins become SUMOylated. Interestingly, we found that synaptic cytosol fractions contained increased levels of free Sumo2 which may further support the notion of highly dynamic SUMOylation at synapses, or non-covalent Sumo2 regulation in these compartments. Ultimately, a dedicated proteomics approach focused on synaptic fractions may be necessary to specifically detect synaptic Sumo2 substrates and uncover specific roles for Sumo2 at synapses(Kieffer et al., 2022; Pronot et al., 2021). Pathway analysis of the Sumo2 dataset revealed that gene ontology terms related to SUMOylation were significantly enriched in the dataset, validating this method to identify potentially SUMOylated substrates. Moreover, pathway analysis of the Sumo1 dataset revealed enrichment of proteins involved in protein transport consistent with roles for Sumo1 in nucleocytoplasmic shuttling(Pichler et al., 2002; Salinas et al., 2004; Westman et al., 2010). Prox1 was validated via Western blot as one of the strongest interactors specific to Sumo2 in the mouse brain. Interestingly, previous studies demonstrated that SUMOylation of Prox1 by Sumo1 occurs *in vitro* and in cancer cell lines(Pan et al., 2009; Shan et al., 2008). During development, Prox1 was found to be SUMOylated by Sumo1, and this interaction is critical for neurogenesis in the neural tube development. However, the role of Prox1 in the mature brain remains unclear, although some data indicate an involvement in adult neurogenesis(Correa-Vázquez et al., 2021). Due to the

extent of the Prox1 and Sumo2 interaction observed in this study, further analysis may uncover key roles of SUMOylated Prox1 in the adult mouse brain. We further found that the RNA binding protein Matr3 interacts with Sumo2 *in vivo*, indicating that even a mild enrichment over WT in our dataset may identify *bona fide* biological interactors. Interestingly, many of the computationally predicted SUMOylation sites in Matr3 (K588 and K843) reside within a structurally disordered region of the protein that also happens to be enriched for mutations causing Amyotrophic Lateral Sclerosis(Johnson et al., 2014). Structurally disordered regions are thought to be critically involved in the formation of protein aggregates in neurodegenerative diseases and given the key role of SUMO in controlling protein solubility, and the recent links between SUMOylation and ALS, SUMOylation of Matr3 may provide key insights into Matr3 biology related to this disease.

SUMOylation occurs in a context-specific manner and can regulate protein localization within cells. One of the best characterized examples concerns the regulation of nucleocytoplasmic shuttling across nuclear pore complexes(Pichler et al., 2002; Salinas et al., 2004; Westman et al., 2010) However, SUMOylation can also regulate sub-compartmental localizations, such as protein targeting to the nucleolus or to heterochromatin(Andreev et al., 2022; Mo et al., 2002; Rawat et al., 2021). As Sumo2 was observed throughout the nucleus and outside the nucleus *in vivo* and in cultured neurons, we sought to determine the subcellular localization of Sumo2 interactions with hits identified via IP-MS using a PLA-based approach (Figure 6). Gtf2i was SUMOylated almost exclusively in the nucleus but interestingly appeared to cluster around heterochromatin. Gtf2i can function as both a transcriptional activator and repressor in response to various signals. For example, the specific splice isoforms β and Δ undergo changes in subcellular localization to differentially regulate the immediate early gene *c-Fos* in response to growth factor signaling(Hakre et al., 2006). However, roles for Gtf2i in regulating heterochromatin remain elusive. SUMOylation

may provide a dynamic mechanism in response to neuronal activity to regulate both targeted expression at the gene level and global transcriptional changes⁵⁹. Finally, interactions between Matr3 and Sumo2/3 occurred broadly throughout the nucleus. However, interactions between Matr3 and Sumo2 in mouse brain occurred at relatively low levels based on immunoprecipitation assays. This discrepancy observed in the nucleus may be explained via non-covalent interactions with Sumo2 through larger protein complexes as Matr3 typically functions in complex with other DNA and RNA binding proteins(Banani et al., 2016; Keiten-Schmitz et al., 2021, 2020). Extranuclear proteins Rangap1, Ctsd, and Kars1, despite having a certain level of PLA signal within the nucleus, displayed an increase in the proportion of cytoplasmic PLA foci relative to nuclear PLA foci implying increased interactions with Sumo2/3 outside the nucleus. Indeed, Rangap1 plays roles in nuclear import, but can also be found to some extent within the nucleus(Cha et al., 2015). PLA foci between Rangap1 and Sumo2/3 were often found near the nuclear membrane, likely at nuclear pore complexes (Figure 6C, inset). Ctsd is a lysosomal protease predominantly localizing to lysosomes, thus interactions between Sumo2/3 and Ctsd may suggest that its SUMOylation may play a role in lysosomal metabolism(Nakanishi, 2003). Finally extranuclear PLA foci could be observed for the lysyl-tRNA Synthetase Kars1, consistent with reports of Sumo interacting with tRNA-related proteins(Chymkowitch et al., 2017; Chymkowitch and Enserink, 2018; Rohira et al., 2013). Together these results suggest that Sumo2 may play a variety of roles outside the nucleus in various subcellular compartments. While PLA assays provide spatial information of putative protein interactions (i.e. interactions occurring within 40 nm), this does not discount indirect interactions (e.g. protein complexes)(Ristic et al., 2016; Sahin et al., 2016). Thus, differentiating between covalent SUMOylation, interactions with SUMO interacting motifs (SIMs), and protein complexes containing SUMOs are not possible using this

approach. Extensive localization and biochemical analyses, including arginine to lysine mutagenesis to block covalent SUMOylation, should be performed to properly assess how SUMO confers regulation on a specific target throughout the cell.

In sum, the novel (His₆-)HA-Sumo2 knock-in mouse lines described here represent powerful tools to facilitate the study of protein SUMOylation via Sumo2 *in vivo*. The HA epitope tag allows for specific yet versatile modes to detect and capture Sumo2 in its native context, without altering its function. Furthermore, these mouse models add to the previously generated His₆-HA-Sumo1 allele, and thus expand the Sumo knock-in mouse toolkit to begin exploring differential SUMOylation in parallel systems. The versatility of these models extends to all other tissue systems, as Sumo proteins are broadly distributed beyond the brain (Hendriks et al., 2018; Uhlén et al., 2015). Beyond basic biology, these alleles can be used to explore disease processes, for example by crossing these mouse lines into disease models, thus enabling the study of the Sumo-linked disease proteome (Stankova et al., 2018). Ultimately, these tools will advance our understanding of the essential biological processes and potential disease targets regulated by SUMOylation.

LIMITATIONS OF STUDY

While the (His₆-)HA-Sumo2 knock-in mice help to overcome technical limitations in studying SUMO biology, including low antibody specificity, inability to differentiate between endogenous SUMO2 and SUMO3, and reliance on overexpression models, it cannot be completely excluded that the knock-in of the (His₆-)HA-tag slightly affects Sumo2 function. Indeed, these mouse lines allow to characterize Sumo1 and Sumo2, but we also observed early lethality on C57BL/6J or C57BL/6N background, which was not seen on an FVB/N background. This indicates an

interaction between the His₆-HA-Sumo2 knock-in genotype and the genetic C57BL/6J or N set-up. Such strain effects are seen in many contexts, highlighting the importance of validating new genetic models using multiple systems and approaches. Furthermore, our observation that only Sumo2, but not Sumo1, is detectable at synapses, does not completely exclude a role for Sumo1 in regulating synapse function *in vivo*.

3.6 Materials and Methods

STAR METHODS

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maxime Rousseaux (max.rousseaux@uottawa.ca).

Materials Availability

HA-Sumo2 and His₆-HA-Sumo2 mouse lines will be provided upon reasonable request.

Data and Code Availability

Mass spectrometry data is available from the lead contact upon request.

Microscopy data reported in this paper will be shared by the lead contact upon request.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Mouse Lines

C57BL/6J

FVB/N (WT)

His₆-HA-Sumo1 (FVB/N Background)

His₆-HA-Sumo2 (FVB/N Background)

HA-Sumo2 (FVB/N Background)

Sex and Age of Experimental Mice

Experiments were performed using a mix of male and female mice to prevent sex biases. Cortical neuron cultures experiments included neurons dissected from individual embryos using a mixture of male and female cultures for all experimental conditions. At the high level of characterization, we do not expect sex effects to be observed however we acknowledge that future in depth analyses may uncover unique sex effects with regards to SUMOylation. All experimental animals were between 8-30 weeks of age. Specific age/sex information for whole brain imaging and mass spectrometry experiments can be found in Supplement Tables S1 and S2.

Mouse Husbandry

His₆-HA-Sumo1 and His₆-HA-Sumo2 mice, kept on an FVB/N background, were housed with up to 5 mice per cage on a 12 h light–dark cycle. Mice were fed *ad libitum* and all husbandry was performed by the uOttawa Animal Care and Veterinary Services staff. All animal work for the His₆-HA-Sumo1 and His₆-HA-Sumo2 mouse lines were done under the breeding (CMMb-3009 and CMMb-3904) protocols approved under the uOttawa Animal Care Committee. Experiments performed on the HA-Sumo2 mice were done in accordance with the guidelines for the welfare of experimental animals issued by the State Government of Lower Saxony, Germany (LAVES). Animals were hosted in a pathogen free facility at the Max Planck Institute of experimental Medicine and were maintained in groups in accordance with European Union Directive 63/2010/EU and ETS 123 (individually ventilated cages, specific pathogen-free conditions, 21 ± 1°C, 55% relative humidity, 12 h/12 h light/dark cycle). Mice received food and tap water *ad libitum* and were provided with bedding and nesting material. Cages were changed once a week.

Animal health was controlled daily by caretakers and by a veterinarian. Health monitoring (serological analyses; microbiological, parasitological, and pathological examinations) was done quarterly according to FELASA recommendations with either NMRI sentinel mice or animals from the colony. The mouse colony used for experiments did not show signs of pathogens. All experiments were performed during a light cycle.

Method Details

His₆-HA-Sumo2 and HA-Sumo2 Mouse Generation

His₆-HA-Sumo2 mice were generated via CRISPR/Cas9-mediated gene editing (Wang et al., 2013). Briefly, an sgRNA targeting the 5' of *Sumo2* was synthesized by direct PCR from pX330 (gift from Zhang lab, Addgene #42230) and in vitro transcribed with the MEGAshortscript T7 Transcription kit (Invitrogen) using the following two primers (forward: 5'-TTAATACGACTCACTATAGGAACCCAAGGTGAGCTCGCGGGTTTTAGAGCTAGAAATAGC-3'; reverse: 5'-AAAAAAGCACCGACTCGGTGCCAC-3'). A single stranded oligodeoxynucleotide (ssODN) was purchased from IDT for homologous-directed recombination to knock-in a His₆-HA-tag into the amino-terminus of Sumo2 (5'-GACCCGGTGCCTCTTTTGTGAAGCGGCAGCTGAGGAGACTCCGGCGCTCGCCATGCCACCACCACCACCACCTACCCATACGATGTTCCAGATTACGCTGCCGACGAGAAACC CAAGGTGAGCTCGCGGCGTCCGGCTGCGCCTACCGTGCGGACCGGTGGGTGGCGCGGCCCGGGGTCCGGCCGAAGCCCGCCGCGG-3'). On the day of injection, Cas9 protein (PNA Bio), sgRNA and repair template (ssODN) were injected into ova from C57Bl/6 female mice and transferred into oviducts of pseudopregnant females. Founder mice were backcrossed at least three times prior to experimentation to get rid of potential off-target mutations. His₆-HA-Sumo2

and His₆-HA-Sumo1 mice were backcrossed at least six times onto an FVB/N background prior to beginning experimentation.

For the generation of the HA-Sumo2 knock-in mouse, two sgRNA, generated via T7 in vitro transcription (Cellscript, #C-MS100625) from corresponding linearized vectors, were used: sgRNA1 sense 5'-GCCTCACCGTGCGGACCGGT-3', sgRNA2 antisense 5'-GCTCACCTTGGGTTTCTCGT-3'. Gibson assembly (NEB #E2611S) was used to generate a double stranded HDR fragment that included the HA tag coding sequence and silent mutations to avoid re-targeting by Cas9 (5'-TTGAGGCTCCCGGGTGGGAAGCTGGTTGATGGGGAAGGACGAAGGGACTGGGAAGTTGATGTGCAGGGGGGAGTTGCATTTGTCCTACTGAACAGGAGTTTTTCTCACAACCCGCTGCTGCTTTTTTTGATGGAGATGCCCCCTTCTACGTGGAGTTGGCCTTCCGTTCTCGACCTAGGCTCACCCACGTCTAAGGCCACGCGCCTTCCTGTCCTCGCCGAGGACCAGCTGGAAGCCATTTTGATTATGCTCCACCAAAGAAGGAGGACAATACAGCCCCGCCAAGAGCCAGGCGCGCCCCCGCGGCGGGACTGGTGCGCGTGTCCGCGCCTCTTCCCGCCGACCTGGCGGGCGCCTGGCCCAGGCGGCGGAGGCGCGGGCCGTGGGGGGGAGGAGGAGGGGTGGTTATGTAAGCGTTGCGCGCTCCCGCGAAGCGGCAGCCAATGAGAAGCCCCGGATGCTCGTGCTCGCGCACGCAGGGCGGGGGGAGGGAAGCGCACGACGTGCGCGCGCCCTCTGCCTCGTCCACCGCTGCCGCCTCCTCCTGCCGCTCCTGGTGCTGCTTGTGCGCTCCCTCAGTGCGGACCCGGTGCCTCTTTTGTGAAGCGGCAGCTGAGGAGACTCCGGCGCTCGCCATGTACCCATACGATGTTCCAGATTACGCTGTCGACGAAAAGCCCAAGGTGAGCTCGCGGCGGCCGGCTGCGCCTCACGGTGCGACCGGTGTGTGGCGCGGCCCGGGGTCCGGCCGATGCCCTCCGCGCGCTTCCCTGCCTGCCCGGGCCCCCACGGGGGCCAGCGGCGGCTCAGGCCCGGCGCTCCGCGCCATTTTCCTCT

CTCGCTCTCTTCCTCCCGCCCGCGCCCGAGGAGGCCCGGCGCCCTCCCCCGCCCCCA
GGCCCTCGGAGCGCGCGCGGCGCGGCGCGAGGCGGGCAGGCGGCGCTTCGCGGGA
GCCCCGCGGGCCCCGGCGGCCCCGCGCTGGCTCCTCGGCGGCGCCCGGCGCGTGTAAG
GAAAGCGTTGGGCGCGCGGGGTCTGGGGCGGCGGCGAAGGCGGGAGTCGTGCGGC
CCGGGGTTTCCCTCTCGGGGCTGGGGCCGCGCCGGATCGGTGGTGTGTGCTCCGGCA
ACCCCGAGTGGGGGATTCCAGAGTCGGTCCGGCTCGAGGCCCAAG-3') (blue, ATG-
coding exon1; red, HA tag coding sequence; green, silent mutations in exon; orange, silent
mutations in intron; underlined, annealing sites for PCR primers used for genotyping). Cas9
protein (NEB #M0646T) and hCas9 mRNA generated via T7 in vitro transcription (Cellscript, #C-
MSC100625) were used. On the day of injection, hCas9 mRNA and protein, the two sgRNAs and
the HDR fragment were injected into zygotes obtained from FVB/N female mice, and injected
zygotes were transferred into oviducts of pseudopregnant females. Founder mice were further
crossed with FVB/N.

Mouse Genotyping

Genomic DNA (gDNA) was isolated from small tail samples collected from mouse embryos
(primary cortical neuron experiments), pups (pre-weaning), or adult mice (endpoint). His₆-HA-
Sumo2 mice were genotyped using parallel reactions with primers targeting the *His₆-HA* knock-in
tag and corresponding genomic loci upstream (Forward: 5'- AGGAAGAGAGCGAGAGAGGAA-
3', Reverse: 5'-CACCACCACTACCCATACGA-3', 224 bp product) and downstream (Forward:
5'-TCGTATGGGTAGTGGTGGTG-3', Reverse: 5'-AGGAGGAGGGGTGGTTATGT-3', 276 bp
product) of the knock-in. 25 µL reactions were prepared using <10ng gDNA, primers (400 nM
final), 1.25µL DMSO (5 % final), and 2X GoTaq (Promega). Thermocycler parameters: 95 °C for

2 min, (95 °C for 30 s, 57.6 °C for 30 s, 72 °C for 40 s) repeated for 35 cycles, final denature at 72°C for 5 min. HA-Sumo2 mice were genotyped with primers flanking the *HA* knock-in tag (Forward: 5'-GCCCTCTGCCTCGTCCAC-3', Reverse: 5'-CCGCCGCGAGCTCACCTTG-3', WT Allele: 160 bp product, KI Allele: 187 bp product). 20 µL reactions were prepared using 1 µL clean gDNA, primers (4 pmol final), and 5X Biozym Hot-Start Taq DNA Polymerase plus extra Mg²⁺ (Biozym 331620XL). Thermocycler parameters: 96°C for 3 min, (94 °C for 30 s, 62 °C for 60 s, 72 °C for 60 s) repeated for 32 cycles, final denature at 72 °C for 7 min. His₆-HA-Sumo1 mice were genotyped as previously described (Tirard et al., 2012).

Biochemical Analysis of Adult Mouse Brain and Spinal Cord

Mice were anesthetized using isoflurane inhalation and sacrificed via decapitation. Brains were quickly isolated, dissected for regional protein analysis, and flash frozen on dry ice. Analysis of total Sumo1 and Sumo2/3 levels were performed on whole brain lysates. Spinal cords were removed using hydraulic extrusion (Richner et al., 2017). Samples were thawed and immediately lysed using a dounce homogenizer in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM sodium chloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) containing 50 mM freshly prepared N-Ethylmaleimide (Sigma), 0.25 % β-mercaptoethanol, and Xpert Protease (GenDEPOT) and Xpert Phosphatase (GenDEPOT) inhibitor cocktails. Whole brain lysates were lysed in 11 mL of RIPA buffer described above and centrifuged at 125,000 x g for 2 hours at 4 °C. Regional lysates were centrifuged at 21,000 x g for 20 minutes at 4 °C and the supernatant was removed and suspended in 4X laemmli buffer (BioRad) with 2-mercaptaethanol (BioRad) and boiled at 95 °C for 5 minutes. Samples were run on 8 % polyacrylamide gels and transferred onto 0.45µm nitrocellulose membranes at 340 mA for 90

minutes and stained with ponceau to normalize protein levels between lysates. Normalized lysates were run on a 4-15 % GTx Mini-PROTEAN (BioRad) or Bolt™ 4-12% Bis-Tris Plus (Invitrogen) gel and transferred onto a 0.45 µm nitrocellulose membrane at 340 mA for 2 hours for western blot analysis. Densitometry was performed using BioRad Image Lab software and FiJi/ImageJ by normalizing the HA intensity to ponceau for High Molecular Weight (>70 kDa) and free Sumo levels.

Real Time Quantitative PCR (RT-qPCR) analysis

RNA was extracted from mouse brain homogenate using Trizol-Chloroform extraction (Invitrogen™ User Guide: TRIzol Reagent version B.0). Briefly, mouse brains were homogenized in 3 ml of PEPI Buffer [5 mM EDTA, 1X protease inhibitor (GenDEPOT cat# P3100-020), in 1X PBS] using a dounce homogenizer. 3 % of homogenate was added to 1 ml of TRIzol Reagent (Fisher Scientific cat# 15-596-026) and RNA was isolated as per the user guide referenced above. For CNS regional RNA analysis, tissue samples were homogenized in 300 µL of PEPI buffer using a pestle and passed through a 28 gauge insulin needle before RNA was extracted using RNeasy Kits (Qiagen cat# 74104). cDNA was synthesized using 5X All-in-One RT Master Mix (Bio Basic cat# HRT025-10) following manufacturer's instructions. RT-qPCR was performed using Green-2-Go qPCR Master Mix (Bio Basic cat# QPCR004-S) with 25 ng cDNA per reaction and primers targeting mouse Sumo paralogs (Wang et al., 2014) *Sumo1* (NM_009460.2) (Forward: 5'-CAGGAGGCAAAACCTTCAAC-3', Reverse: 5'-CTCCATTCCCAGTTCTTTTCG-3'), *Sumo2* (NM_133354.2) (Forward: 5'-ACGAGAAACCCAAGGAAGGA-3', Reverse: 5'-CTCCATTTCCTCAACTGTGCAG-3'), *Sumo3* (NM_001301672) (Forward: 5'-AGAAGCCCAAGGAGGGTGT-3', Reverse: 5'-CCTCGGGAGGCTGATCCT-3') and

housekeeping controls *Gapdh* (NM_001289726) (Forward: 5'-GGAGAGTGTTTCCTCGTCCC-3', Reverse: 5'- ATGAAGGGGTCGTTGATGGC-3') and *Hprt1* (NM_013556.2) (Forward: 5'-TGATAGATCCATTCCTATGACTGTAGA-3', Reverse: 5'-AAGACATTCTTTCCAGTTAAAGTTGAG-3'). Reactions were run on BioRad CFX96 thermocycler (protocol: 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 60 s, then melting curve). *Sumo* Ct values were standardized to the average of *Gapdh* and *Hprt1* Ct values.

Brain preparation for whole brain clearing and HA immunolabeling.

Mice were sedated via intraperitoneal injection of 16.25 mg sodium pentobarbital (Euthanyl, DIN 00141704). Once sedated, mice were perfused using 10 mL 1X PBS + 10 U/mL heparin (Millipore Sigma, H3393-50KU) followed by 10 mL of freshly prepared 4 % paraformaldehyde. Brains were carefully isolated and stored in 13 mL of 4 % paraformaldehyde at 4 °C overnight with gentle shaking. Brains were rinsed with 1X PBS then shipped to LifeCanvas Technologies (MA) in 1X PBS + 0.02 % sodium azide.

Whole mouse brain processing, staining, and imaging

Whole mouse brains were processed using the SHIELD protocol (LifeCanvas Technologies(Park et al., 2018)). Samples were cleared for 1 day at 42 °C then actively immunolabeled using SmartBatch+ (LifeCanvas Technologies) based on eFLASH technology integrating stochastic electrotransport (Kim et al., 2015) and SWITCH (Murray et al., 2015). Each sample was labeled with 60 µg anti-NeuN (Encor, MCA-1B7) and 36 µg rabbit anti-HA-tag (Cell Signalling Technology #3724) followed by fluorescently conjugated secondary antibodies in a 3:2 primary:secondary molar ratio (Jackson ImmunoResearch). Samples were incubated in EasyIndex

(LifeCanvas Technologies) for refractive index of $RI = 1.52$ and imaged at 3.6X using a SmartSPIM axially-swept light sheet microscope (LifeCanvas Technologies). Images were tile corrected, de-striped, and registered to the Allen Brain Atlas (Allen Institute: <https://portal.brain-map.org/>). NeuN channels for each brain were registered to 8-20 atlas-aligned reference samples using successive rigid, affine, and b-spline warping (SimpleElastix: <https://simpleelastix.github.io/>). Average alignment to the atlas was generated across all intermediate reference sample alignments to serve as the final atlas alignment value per sample. Fluorescent measurements from the acquired images were projected onto the Allen Brain Atlas to quantify the total fluorescence intensity per region defined by the Allen Brain Atlas. These values were then divided by the volume of the corresponding regional volume to calculate the intensity per voxel measurements.

HA Immunoprecipitation from mouse brain lysates

Immunoprecipitation was performed based on the previously established protocol (Tirard et al., 2012; Tirard and Brose, 2016). Briefly, mice aged 9-16 weeks old were anesthetized and sacrificed via decapitation and the brain was quickly removed and homogenized using a dounce homogenizer in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM sodium chloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) containing 50 mM freshly prepared N-Ethylmaleimide (Sigma), 0.25 % β -mercaptoethanol, and Xpert Protease (GenDEPOT) and Xpert Phosphatase (GenDEPOT) inhibitor cocktails and boiled for 5 minutes at 95 °C. Samples were lysed on ice for 20 minutes with vigorous vortexing every 5 minutes before ultracentrifugation at an average of 100,000 x g for 30 minutes at 4 °C. Supernatants were removed and spiked with 50 mM N-ethylmaleimide before being added to 50 μ L of magnetic protein G

Dynabeads (Invitrogen) pre-conjugated with 10 µg mouse HA antibody (in house) per sample. Samples were then placed on rotator at 4 °C for 1 hour. Beads were washed three times in 10 mL of RIPA buffer containing 20 mM N-Ethylmaleimide, protease, and phosphatase inhibitors. Wash buffer was thoroughly removed, and beads were eluted using 75 µg synthetic HA peptide (Sino Biological Inc.).

Sample Preparation for Mass Spectrometry

Two thirds of eluted protein sample from HA Immunoprecipitation were run on a 4-15% Mini-PROTEAN (Bio-Rad) gel to separate proteins and remove synthetic HA peptide. Samples were stained using Silver Stain for Mass Spectrometry Kit (Thermo Scientific, 24600). Lanes were cut into 6-7 gel slices per sample and stored in 1% acetic acid until analysis via LC-MS/MS.

Protein Identification by LC-MS/MS

Proteomics analysis was performed at the Ottawa Hospital Research Institute Proteomics Core Facility (Ottawa, Canada). Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko (Shevchenko et al., 2006) but without the use of iodoacetamide for cysteine alkylation due to the treatment of 50 mM N-ethylmaleimide (Sigma) during the sample immunoprecipitation. Peptide extracts were concentrated by Vacufuge (Eppendorf). LC-MS/MS was performed using a Dionex Ultimate 3000 RLSC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). MASCOT software version 2.7.0 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The observed spectra were matched against *Mus musculus* sequences from SwissProt (version 2021-02) and against an in-house database of common contaminants. The results were exported to Scaffold

(Proteome Software, USA) for further validation and viewing and will be uploaded to the ProteomXchange Consortium.

Gene Ontology analysis

Top His₆-HA-Sumo1 and His₆-HA-Sumo2 interactors were analyzed using gProfiler2 web tool (<https://biit.cs.ut.ee/gprofiler/gost>) (Peterson et al., 2020). Lists were arranged in descending order based on relative peptide abundance and analyzed as an ordered query for *Mus musculus* proteins using Benjamini-Hochberg FDR significance threshold with an alpha of 0.05.

Immunohistochemistry of Mouse Brain

Mice were anesthetized with 30 µl of 120 mg/kg Euthanyl (DIN00141704) and then perfused with 10 ml 1x phosphate buffered saline (PBS) and 10 ml 4 % paraformaldehyde (PFA). Brain and spinal cord tissue were collected and stored for 48 hours in 4 % PFA. Brain and spinal cord tissue were then dehydrated in 10 %, 20 % and 30 % sucrose solutions for 48 hours each before being flash frozen in -40 °C isopentane for 1 minute. Tissues were then sectioned at 20 µm and -21 °C on the Thermo Scientific HM 525 NX cryostat at the Louise Pelletier Histology Core at the University of Ottawa and stored free floating in 1x PBS + 0.02 % NaN₃ at 4 °C until use in staining. Brains tissue were incubated for 24 hours in blocking buffer (1.5 % Triton X-100, 5 % cosmic calf serum in 1X PBS), 24 hours in primary antibody (1:500 HA-tag C29F4 rabbit monoclonal antibody (Cat: 3724S, Cell Signaling Technology), and 1 hour in secondary antibody (1:500 Alexa Fluor 568 donkey anti mouse antibody, Cat: A10037, Lot: 1917938)) with DAPI (1:1,000 Millipore Sigma, D9542-1MG). Tissue was washed 5 times for 5 minutes each in 1x PBS between each

treatment and mounted on Fisherbrand Superfrost Plus slides. Slides were left to dry for 24 hours and covered with DAKO mounting medium (Cat: S3023, Lot: 11347938) and #1.5 coverslips. For the analysis of the synaptic localization of Sumo2, HA-Sumo2 mouse brains were used (Figure S1A). Mice were anaesthetized (250 mg/Kg Avertin i.p.) and transcardially perfused with PBS and then with 4 % (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 at 4 °C for 10 min. Brains were removed and post-fixed for 1 h at 4 °C. The tissue was cryoprotected in 30 % (w/v) sucrose in phosphate-buffered saline (PBS). Brains were frozen in the cryostat and sagittal 35 μ m sections were prepared with a cryostat and collected free-floating in PBS. For immunohistochemistry, sections were pre-incubated for 24 h in PB containing 3 % horse serum (HS), 3 % fish skin gelatin (FSG), and 0.3 % Triton X-100, and were then incubated for 3 days at 4 °C in primary antibodies diluted in PBS containing 3 % HS, 3 % FSG and 0.3 % Triton X-100. After washing repeatedly in PBS and overnight, sections were incubated overnight in dye coupled secondary antibodies, repeatedly washed, and mounted on slides with Aquapolymount (Polysciences). The antibodies used are listed in Table S3.

Primary Cortical Neuron Cultures

Pregnant mice were euthanized between at gestation E14.5-15.5 with 48 mg Pentobarbital Sodium (Bimeda-MTC, 8015E) delivered via intraperitoneal injection. Embryos were removed, washed in chilled PBS (Wisent Bioproducts, 311-010-CL), and cortices were isolated in chilled HBSS (Sigma Aldrich, H9394). Cortices were dissociated for 20 minutes with trypsin (Thermo Scientific, 90305) at 37 °C before adding trypsin inhibitor and DNase solution to quench reaction. Cells were pelleted at 2,500 x g for 5 minutes at 4 °C and washed with trypsin inhibitor with DNase solution. Cortical neurons were pelleted at 2,500 x g for 5 minutes at 4 °C and resuspended in 1 mL Neurobasal

outgrowth media (Thermo Scientific, 21103049), supplemented with B-27 (Thermo Scientific, 17504044), N-2 (Thermo Scientific, 21103049), 500 μ M L-Glutamine (Wisent Bioproducts, 609-065-EL), and 0.5 % penicillin/streptomycin (GE Healthcare Life Sciences, SV30010) before plating. Cultures were maintained for 7 days in vitro with a half media change after 3-4 days.

Immunofluorescence in Primary Cortical Neurons

Micro Coverglass #1.5 coverslips (Electron Microscopy Sciences) coverslips were pre-coated with poly-D-lysine (50 μ g/ml) overnight at 37 °C, then washed with distilled water three times and air-dried at room temperature for at least two hours. Primary mouse cortical neurons were seeded at 75,000 cells per coverslip and cultured as described in Primary Cortical Neuron Cultures. On day 7, neurons were fixed using 10 % phosphate buffered formalin for (Fisher Chemical, SF100-4) for 10 minutes followed by 3 x 5-minute washes in 1 mL of 1X PBS. Neurons were blocked in 500 μ L of blocking buffer (1.5 % Triton X-100, 10 % cosmic calf serum in 1X PBS) for 1 hour, then incubated in 300 μ L of primary antibody diluted in blocking buffer overnight at 4 °C. The following day, the neurons were washed for 4 x 5-minute washes in 1 mL of 1X PBS then incubated in 300 μ L of secondary antibody diluted in blocking buffer for 2 hours at room temperature. Next, the neurons were washed for 4 x 5-minute washes in 1 mL of 1X PBS, dried, and then placed on slides with Vectashield Antifade Mounting Medium with DAPI (MJS Biolynx Inc., H-1200). Z-Stack images were obtained on a Zeiss AxioObserverZ1 LSM800 Confocal Microscope at 63X magnification through a Z distance of 10 μ m per image using optimal 0.27 μ m spacing per slice. The dimensions were set to 1,024 x 1,024 pixels.

Proximity Ligation Assay in Primary Cortical Neurons

Primary mouse cortical neurons were cultured and fixed as described for immunofluorescence experiments. Individual coverslips for Proximity Ligation Assay experiments (PLA) were transferred to 12-well plates and outlined with a hydrophobic pen. Neurons were blocked using 40 μ L of Duolink blocking buffer (Sigma Aldrich, DUO82007) at 37 °C for 1 hour and were then washed for 3 x 5 minutes washes in 1 mL 1X PBS. Next, the neurons were incubated in 300 μ L of primary antibody diluted in blocking buffer (1.5 % Triton X-100, 10 % cosmic calf serum in 1X PBS) overnight at 4 °C. The following day, the neurons were washed for 3 x 5 minutes washes in 1 mL of Duolink Wash Buffer A (0.01 M Tris-Base, 0.15 M NaCl, 0.05 % Tween-20, pH 7.4) followed by incubation in 40 μ L of Duolink PLA MINUS (Sigma Aldrich, DUO82004) and PLUS probes (Sigma Aldrich, DUO82002) at 37 °C for 1 hour. Duolink PLA probes were diluted in antibody diluent (Sigma Aldrich, DUO82008) at a 1:5 dilution. Neurons were washed for 3 x 5 minutes washes in Duolink Wash Buffer A and then incubated in 40 μ L of ligase (Sigma Aldrich, DUO82027) at 37 °C for 30 minutes. Ligase was diluted in 1X ligation buffer (Sigma Aldrich, DUO82009) at a 1:40 dilution. Neurons were washed for 3 x 5-minute washes in Duolink Wash Buffer A and then incubated in 40 μ L of polymerase (Sigma Aldrich, DUO82028) at 37 °C for 90 minutes. Polymerase was diluted in 1X amplification buffer (Sigma Aldrich, DUO82011) at a 1:80 dilution. Then, neurons were washed 2 x 10 minutes in Duolink Wash Buffer B (0.2 M Tris-Base, 0.1 M NaCl, pH 7.5) and then again in 1 mL of Duolink Wash Buffer B diluted at 1:100 for 1 minute. Coverslips were briefly air dried and then mounted on slides using Vectashield Antifade Mounting Medium with DAPI. Z-Stack images were obtained on a Zeiss AxioObserverZ1 LSM800 Confocal Microscope at 63X magnification with a 5X digital zoom through a Z distance of 10 μ m per image using optimal 0.27 μ m spacing per slice with dimensions set to 512 x 512

pixels. Images were analyzed and quantified using the Spots function on the Imaris (ver. 9.9.1 Bitplane, Switzerland) software. Localization of the foci (nuclear versus cytoplasmic) was determined using the Orthogonal Views function for colocalization with DAPI signal.

Tissue Lysis

Dissected mouse organs were lysed in 1X RIPA buffer (9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1X protease inhibitor (GenDEPOT, P3100), 1X phosphatase inhibitor (GenDEPOT, P3200) and 50 mM N-ethylmaleimide (Sigma-Aldrich, E3876-5G) using a Dounce homogenizer. Tissue lysates were centrifuged at ~21,000 x g for 15 minutes at 4°C and the resulting supernatant was transferred into a fresh microcentrifuge tube.

Subcellular fractionation

Western blot analysis of brain subcellular fraction was performed as described previously (Jones and Matus, 1974; Tirard et al., 2012).

Western blot analysis

SDS-PAGE was performed with standard discontinuous gels or with commercially available 4%-12% Bis-Tris gradient gels (Invitrogen). Western blots were probed using primary and secondary antibodies as indicated in Table S3. Blots were routinely developed using enhanced chemiluminescence (GE Healthcare) and imaged using an INTAS ECL Chemostar PLUS Imager HR 6.0. For quantitative Western blotting, transferred proteins were visualized using the total protein stain MemCode (Invitrogen). Western blot signals were visualized using an INTAS ECL

Chemostar PLUS Imager HR 6.0 apparatus and quantified using ImageJ. For normalization, a ratio of the antibody signal relative to the total protein stain as revealed by the MemCode was performed.

Confocal Imaging and Image analysis

MAP2, HA-Sumo2, DAPI and the respective synaptic marker fluorescence intensities were acquired from the hippocampal CA3 pyramidal layer and the *stratum radiatum*. Multi-channel z-stacks of 338 x 338 μm -sized fields of view were acquired with a 40x oil immersion objective (1.3 NA) of a Nikon Eclipse Ti confocal microscope equipped with a Yokogawa W1 spinning disk and an Andor iXon Ultra 888 EMCCD camera. A custom macro written within the FIJI software package (Schindelin et al., 2012) automatically identified the z-plane of highest HA-Sumo2 intensity, out of which all channels were extracted. The DAPI, MAP2 and synaptic marker channel of this z-plane were segmented with the Trainable WEKA Segmentation FIJI plugin (Arganda-Carreras et al., 2017), resulting in binary masks (Figure 3A, bottom row) that were subsequently used to extract the respective HA-Sumo2 mean intensity.

To more closely investigate HA-Sumo2 colocalization with the respective synaptic markers, high-resolution, multi-channel fields of view (x, y: 30, 30 μm) of the respective stains within the Mossy fiber region were acquired using 40x oil-immersed objective (NA=1.4) of a Zeiss LSM 880 confocal laser scanning microscope. Oversampled (x, y, z: 30, 30, 100 nm), 1.2 μm -sized z-stacks within the mossy fiber region were acquired for each fluorescent channel. Signal to noise ratio and resolution were subsequently enhanced and sample geometry was corrected with the SVI Huygens deconvolution software package (ver. 22.04, Hilversum, Netherlands). To obtain object-based colocalization information, surfaces depicting either HA-Sumo2 or the respective marker were

generated with an Imaris (ver. 9.9.1 Bitplane, Switzerland) batch workflow, which automatically and identically processed the three-dimensional fields-of-view originating from WT or knock-in animals. All HA-Sumo2 and the respective synaptic marker surface objects were created, yielding the total object number for the respective stain (Figure S5- top and middle rows of A and B). Synaptic marker objects were designated as collocating with the HA-Sumo2 objects only if they overlapped with HA-Sumo2 objects and if they contained a minimum HA-Sumo2 average intensity (Figure S5, bottom rows of A and B). Data resulting from both analysis workflows were assembled and quantified with the KNIME software package (ver. 4.5.2, Zurich, Switzerland).

Quantification and Statistical Analysis

Statistical tests were performed using PRISM 9.4. Test type was picked based on the number of comparisons made. Levels of statistical significance are indicated in figure legends.

3.7 Acknowledgments

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3.8 Supplementary Information

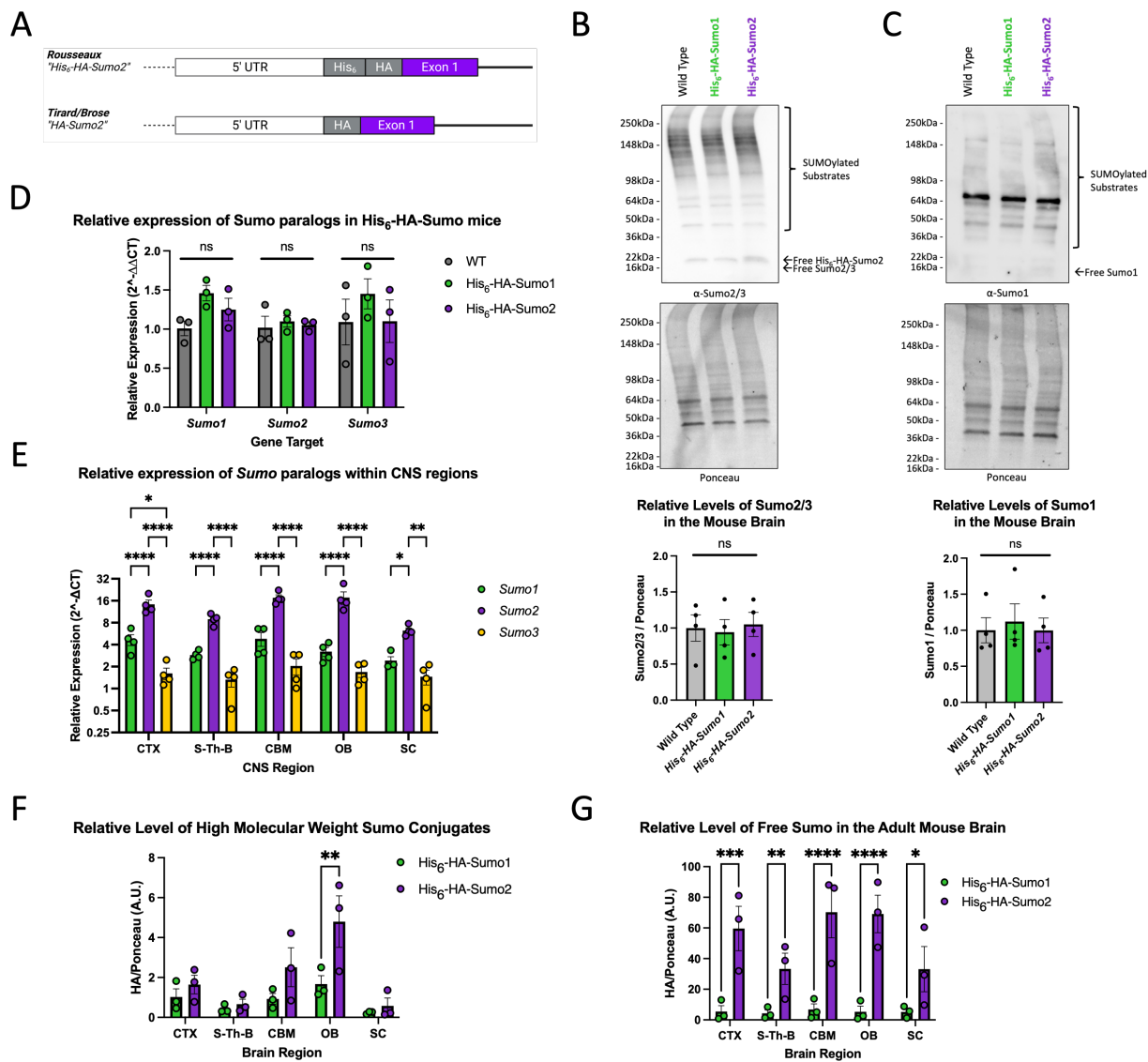
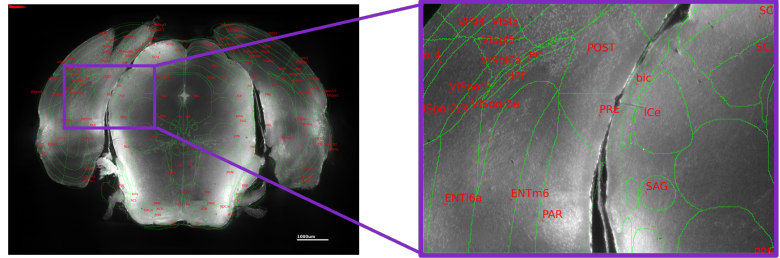
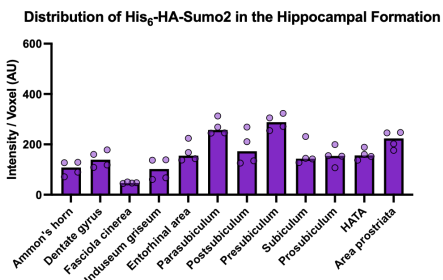


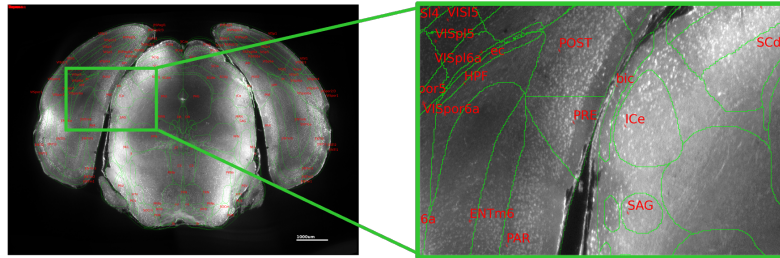
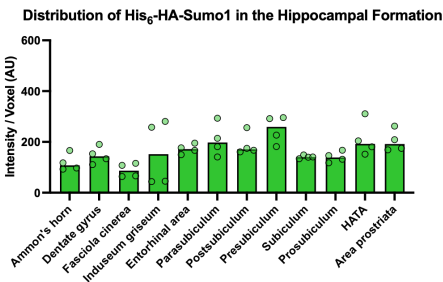
Figure S1: Heterozygous His₆-HA-Sumo2 and His₆-HA-Sumo1 mice exhibit normal Sumo levels. Related to Figure 1.

(A). Schematic of His₆-HA-Sumo2 and HA-Sumo2 knock-in mice. (B). Anti-Sumo2/3 Western blot analysis of total brain homogenates from heterozygous His₆-HA-Sumo1, His₆-HA-Sumo2 mice and WT controls. Anti-Sumo2/3 signal (bracket on the right side of the top left panel) was analyzed by densitometry. Signal from either heterozygous His₆-HA-Sumo1 or His₆-HA-Sumo2 mice was normalized to ponceau signal (N=4). (C). Anti-Sumo1 Western blot analysis of total brain homogenates from heterozygous His₆-HA-Sumo1, His₆-HA-Sumo2 mice and WT controls. Anti-Sumo1 signal (bracket on the right side of the top left panel) was analyzed by densitometry. Signal from heterozygous His₆-HA-Sumo1 and His₆-HA-Sumo2 mice was normalized to ponceau signal (N=4). (D). RT-qPCR analysis of *Sumo1*, *Sumo2*, and *Sumo3* transcript levels in whole brain from heterozygous His₆-HA-Sumo1, His₆-HA-Sumo2 mice and WT controls (N=3). (E). Alternative comparison/data presentation for regional *Sumo1*, *Sumo2*, or *Sumo3* expression displaying changes in *Sumo* paralog expression per region (N=4). (F). Alternative comparison/data presentation of Western blot analysis of free Sumo levels per region. (G). Alternative comparison/data presentation of Western blot analysis of conjugated Sumo levels per region. All statistical tests were analyzed using an ordinary One or Two-way ANOVAs with Tukey's multiple comparisons tests. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

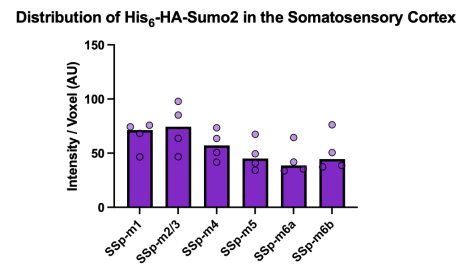
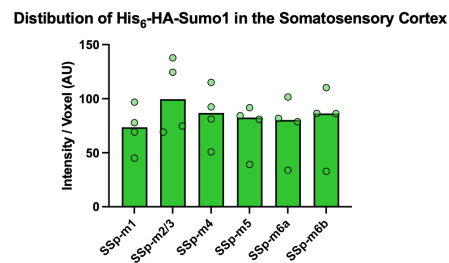
A



B



C



D

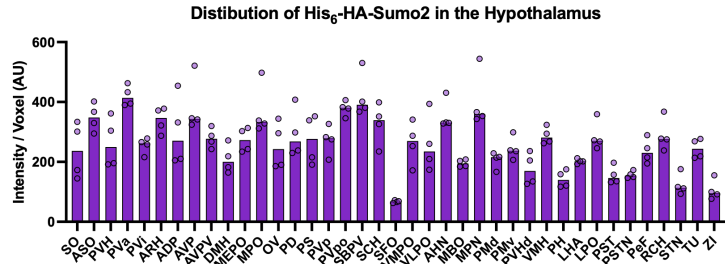
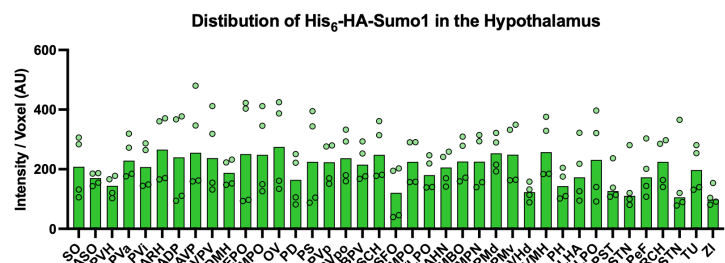


Figure S2: Detailed examination of regional anatomical Sumo paralog distribution. Related to Figure 2.

(A). Bar plot depicting the relative levels of anti-HA immunosignal in the regions of His₆-HA-Sumo2 hippocampus. Each datapoint is the mean intensity from a single hemisphere (N=2, two hemispheres/replicate). Middle and Left panels depict a representative image and zoom of the hippocampal formation overlayed with a mask depicting anatomical regions defined by the Allen Brain Atlas. Scale Bar: 1000 μ m. **(B).** Bar plot depicting the relative levels of anti-HA immunosignal across His₆-HA-Sumo1 brain regions. Middle and Left panels depict a representative image and zoom of the hippocampal formation overlayed with a mask depicting anatomical regions defined by the Allen Brain Atlas. Scale Bar: 1000 μ m. **(C).** Bar plot depicting relative anti-HA immunosignal levels in the primary somatosensory area–mouth (SSp-m) layers of His₆-HA-Sumo1 (Top) and His₆-HA-Sumo2 (Bottom) brain **(D).** Bar plot depicting relative anti-HA immunosignal levels in regions of the hypothalamus of His₆-HA-Sumo1 (Top) and His₆-HA-Sumo2 (Bottom). Each datapoint from the brain atlases depict the mean intensity from a single hemisphere (N=2, two hemispheres/replicate). For each abbreviated brain region, refer to Table S1 for legend. Data are presented as mean $\pm$ SEM.

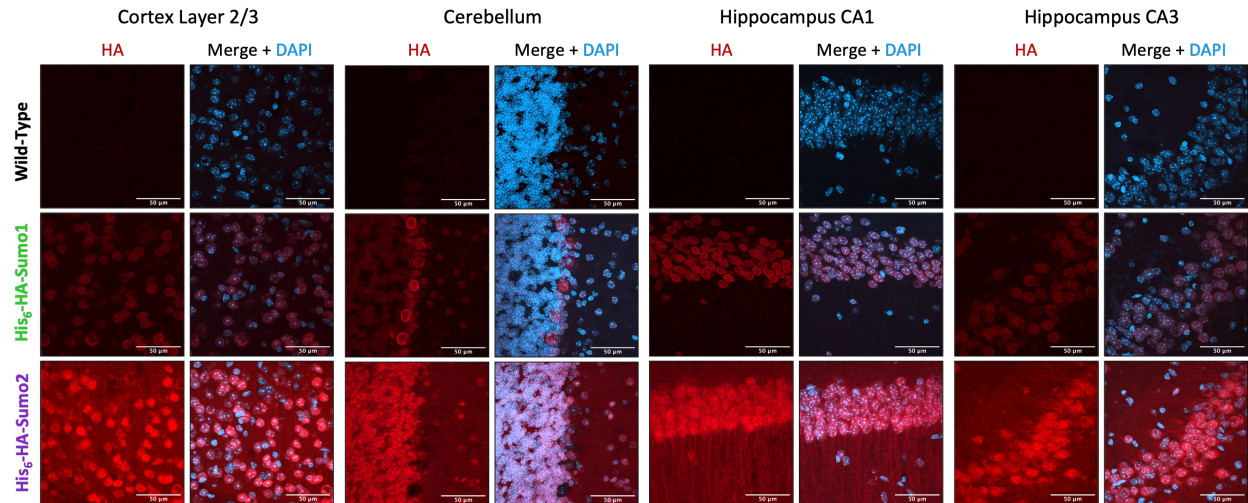


Figure S3: Sumo1 and Sumo2 share similar and distinct anatomical locations and subcellular compartments. Related to Figure 2.

Confocal microscopy analysis of anti-HA (red) and DAPI (blue) nuclear labeling of wild-type (untagged, top lane), His₆-HA-Sumo1 (middle lane) and His₆-HA-Sumo2 (bottom lane) cortex layer 2/3, cerebellum, and hippocampal CA1 and CA3 regions. Z-projection images are representative of three independent replicates. Scale bar: 50 μm

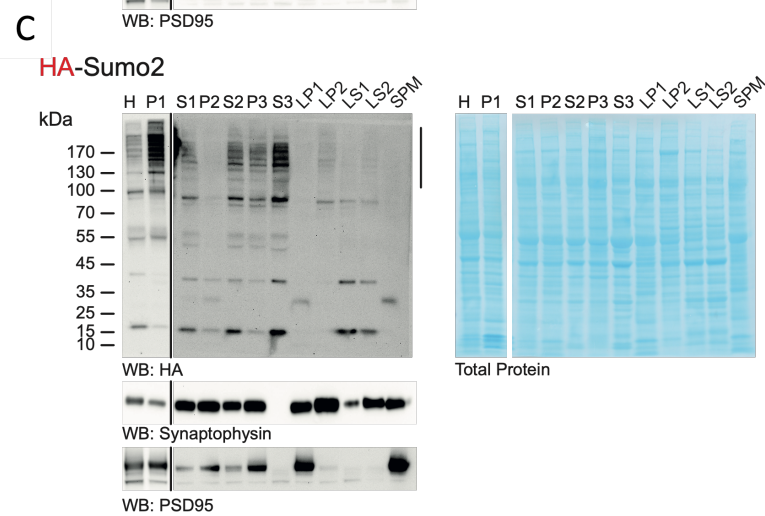
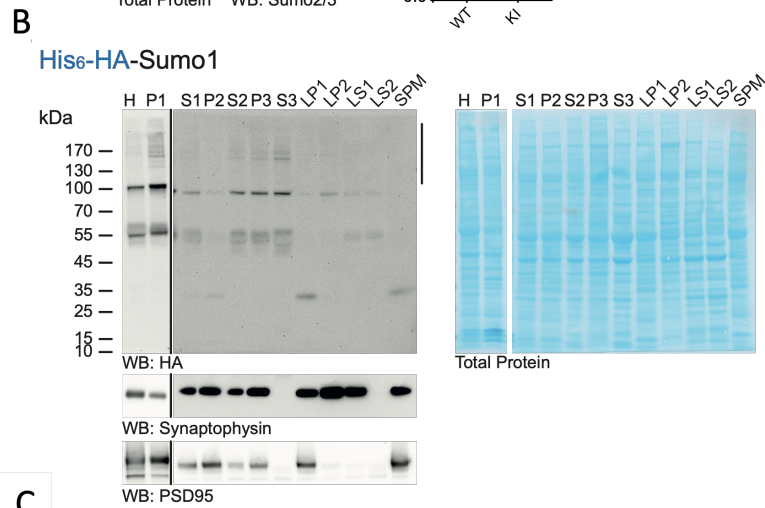
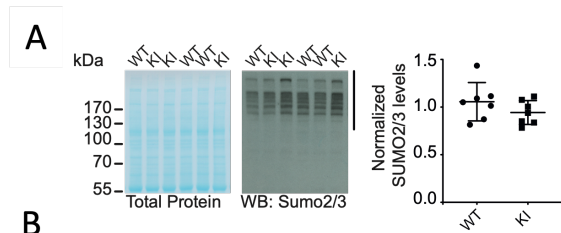


Figure S4: Differential levels and sub-cellular distribution of Sumo1 and Sumo2 conjugates in whole mouse brain fractions. Related to Figure 3.

(A). Total Protein stain (left panel) and anti-HA Western blot (right panel) analysis of whole brain lysates from homozygous HA-Sumo2 and corresponding WT littermates. Anti-HA signal (black bar on the right) indicates Sumo2 signal was normalized to the total protein stain and quantification is shown by the dot plot (right panel). (B and C). Total protein stain (right panel) and anti-HA (left panel) Western blot analysis of subcellular fractions from homozygous His₆-HA-Sumo1 (B) and HA-Sumo2 KI (C). Western blot analysis using anti-Synaptophysin and PSD95 (bottom panels) validates the subcellular fractionation procedure. Anti-HA signal (black line on the right of each blot) was normalized to the total protein stain and quantification is depicted as a bar plot (right panel). The black line between the HA panel indicates that different exposure times are depicted, as the HA signal in the P1 fraction saturates faster than in the other fractions. Thus, the color of the first two bars is lighter to indicate that the quantification was done after different exposure time. Subcellular fractions are designated as follows: *H*, whole brain homogenate; *P1*, nuclear pellet; *P2*, crude synaptosomal pellet; *P3*, light membrane pellet; *S3*, cytosolic fraction; *LPI*, lysed synaptosomal membranes; *LP2*, crude synaptic vesicle fraction; *LS2*, cytosolic synaptosomal fraction; *SPM*, synaptic plasma membranes. Bar plots (Right) depict the quantification of anti-HA signal as indicated by the black line on the right side of each anti-HA Western blot in C and D, relative to the total protein stain. Data are presented as mean $\pm$ SEM.

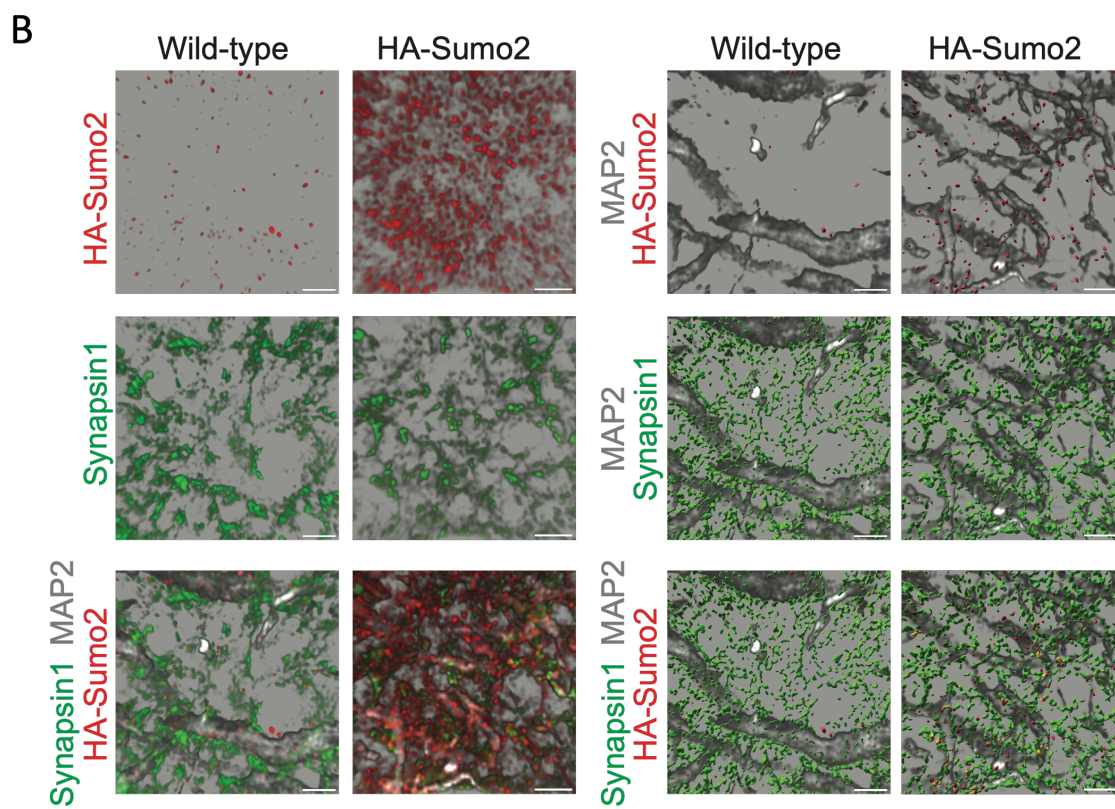
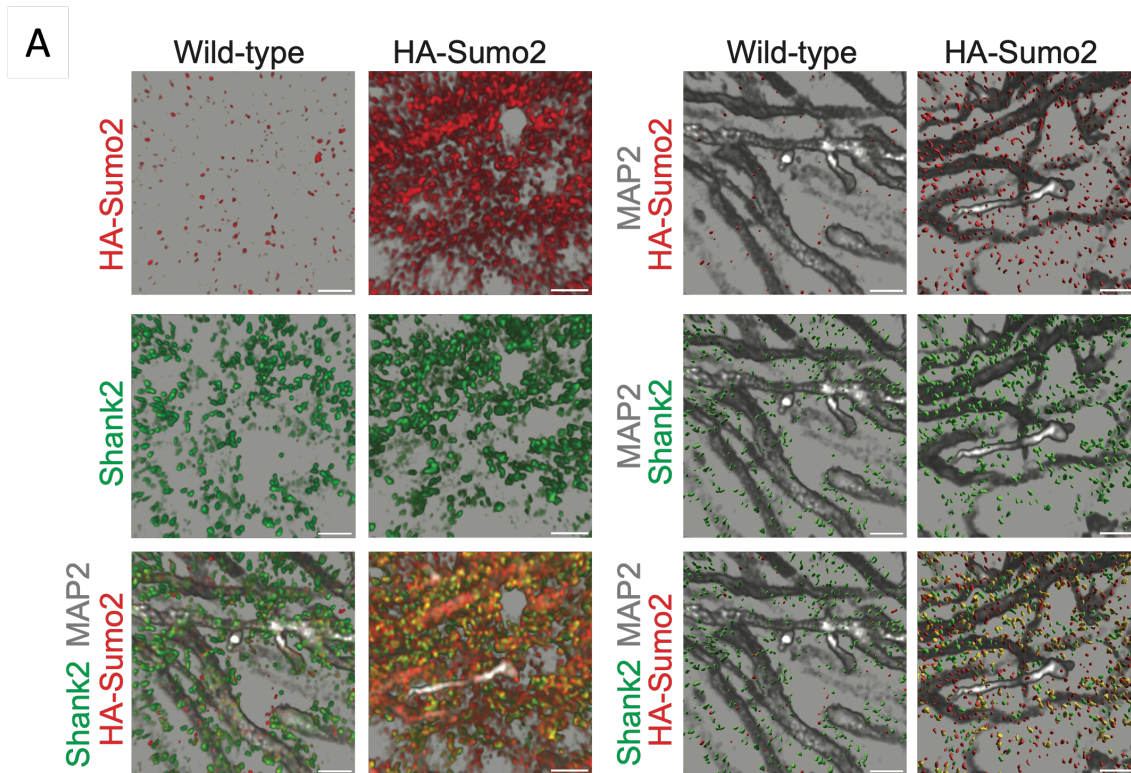


Figure S5: Imaris-based representation of the synaptic Sumo2 co-localization with Shank2 and Synapsin1. Related to Figure 4.

Blended representation generated in Imaris using images from Figure 4B and depicting the colocalization between HA-Sumo2 and Shank2 (**A**) and Synapsin 1 (**B**). The two left panels depict the individual HA-Sumo2 (red, top row A and B), Shank2 (green, middle row A) and Synapsin1 (green, middle row B) raw signal, as well as an overlay thereof (bottom rows, A and B), which additionally contains the MAP2 (grey) raw signal. In all cases, the lookup table of the respective channel was scaled equally to enable direct visual comparison of channel intensity between WT and HA-Sumo2 KI. The right two panels depict the raw MAP2 signal (grey). Instead of the raw signal, Imaris-generated, 3D surface objects based on HA-Sumo2 (red surfaces, top row A and B), Shank2 (green surfaces, middle row A) or Synapsin1 (green surfaces, middle row B) signals are shown. Yellow, surface objects representing colocalization with HA-Sumo2 are shown in the respective bottom rows of A and B, along with non-colocalizing Shank2 (green, bottom row A) or Synapsin1 (green, bottom row B) or non-colocalizing HA-Sumo2 surfaced (red, bottom rows A and B). Scale bar: 5 μm .

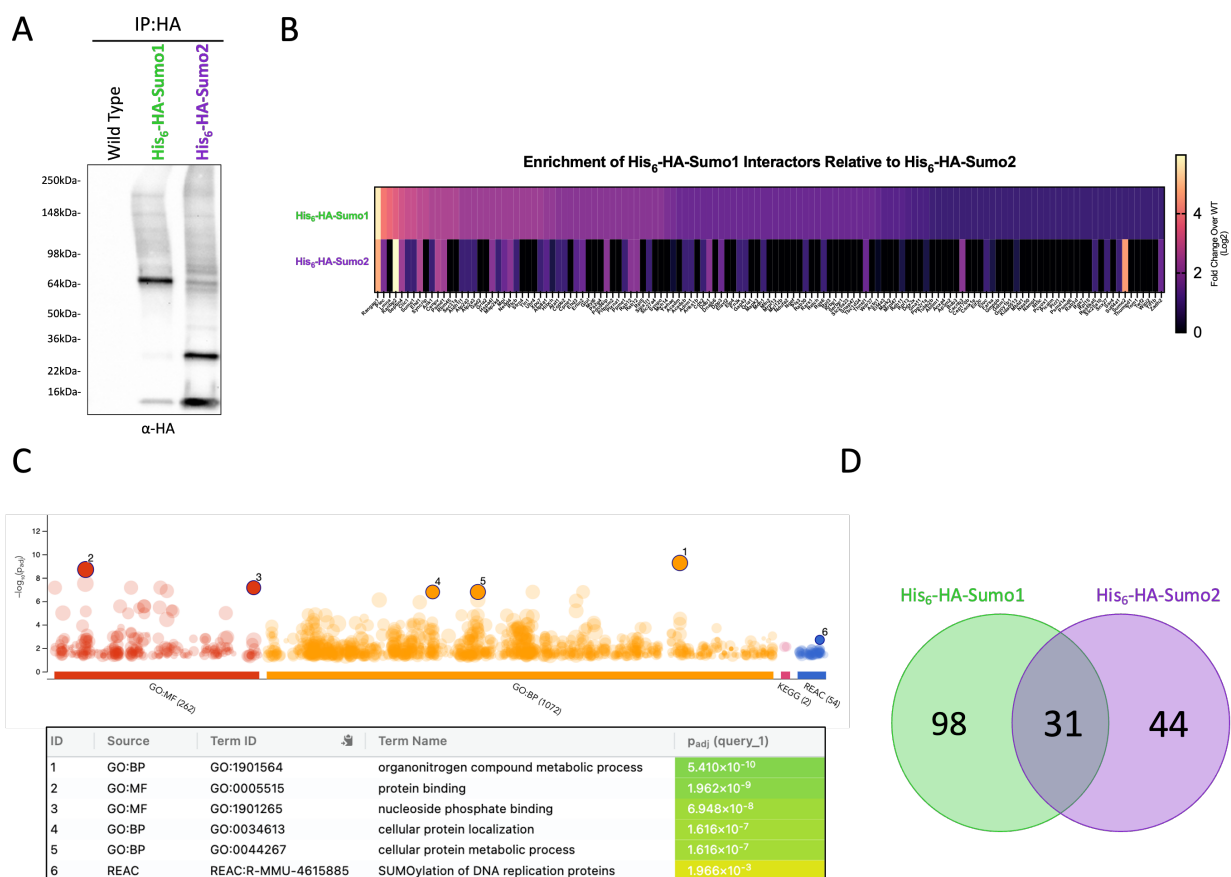


Figure S6: Neuronal Sumo1 has shared and distinct substrates compared to Sumo2 *in vivo*.

Related to Figure 5.

(A). Anti-HA Western blot analysis of eluates from anti-HA affinity immunoprecipitation (IP: HA) from WT, His₆-HA-Sumo1, and His₆-HA-Sumo2 mouse brain lysates demonstrating HA signal patterns of Sumo1 and Sumo2 and their corresponding conjugates **(B)**. Heat map depicting the relative peptide abundance of His₆-HA-Sumo1 interactors relative to levels in His₆-HA-Sumo2 immunoprecipitation. **(C)**. gProfiler2 Gene Ontology analysis for His₆-HA-Sumo1 interactors. **(D)**. Venn diagram depicting unique and common His₆-HA-Sumo1 and His₆-HA-Sumo2 interactors.

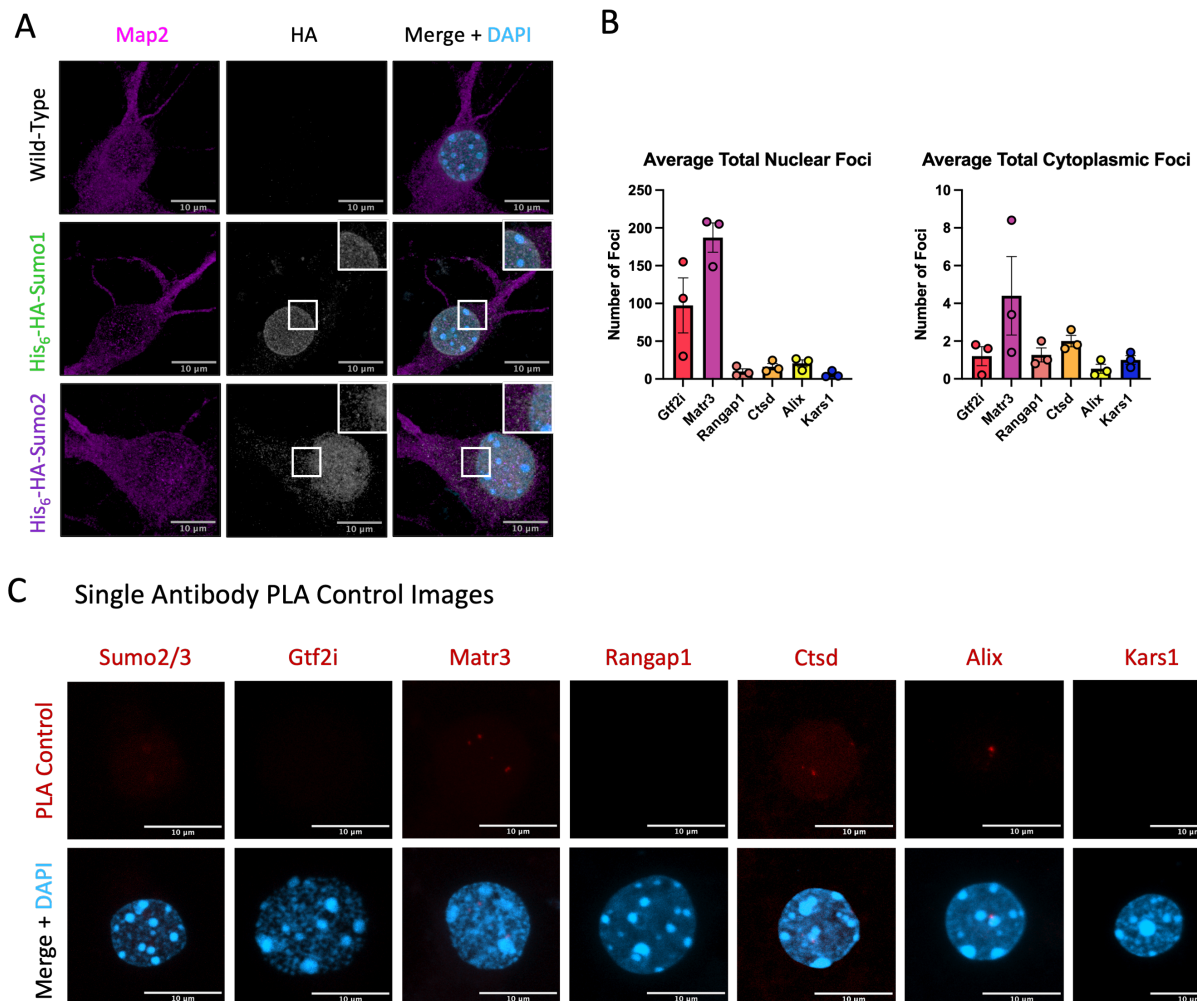


Figure S7: Localization of Sumo2 to extranuclear compartments and proof of PLA assay specificity. Related to Figure 6.

(A). Confocal microscopy analysis of anti-Map2 (purple) and anti-HA (grey) immunolabeling of WT (top lanes), His₆-HA-Sumo1 (middle lanes), and His₆-HA-Sumo2 primary cortical neurons (bottom lanes). Scale bar: 10 μ m. N=3 **(B).** Bar plot depicting the total number of nuclear (left plot) and cytoplasmic (right plot) PLA foci for the indicated antibody (N=3). Data are presented as a mean $\pm$ S.E.M. **(C).** Representative Z-projected images of proximity ligation assays performed with only a single antibody (indicated on top, top lanes) merged with DAPI (bottom panels). Scale bar: 10 μ m. Data are presented as mean $\pm$ SEM.

Table 1: Whole Brain Imaging Analysis – Related to Figure 2

Due to size and complexity, table can be found online:

<https://doi.org/10.1016/j.isci.2023.106350>

Table 2: Mass Spectrometry Analysis – Related to Figure 5

Due to size and complexity, table can be found online:

<https://doi.org/10.1016/j.isci.2023.106350>

Table 3: Antibody List – Related to STAR Methods

Primary Antibody Target	Type	Host Species	Company	Catalog Number	Concentration
Alix	Primary	Rabbit	Protein Tech Group	12422-1-AP	1:500 (ICC, PLA)
Ctsd	Primary	Rabbit	Protein Tech Group	21327-1-AP	1:500 (ICC, PLA)
Gapdh	Primary	Mouse	Protein Tech Group	600004-1-Ig	1:100000 (WB)
Gephyrin	Primary	Mouse	Synaptic Systems	147111	1:1000 (IHC)
Gtf2i	Primary	Rabbit	Protein Tech Group	10499-1-AP	1:500 (ICC, PLA)
HA	Primary	Goat	Novus	BP600-362	1:1000 (IHC)
HA	Primary	Mouse	In House	N/A	5-10µg/brain (IP)
HA (Clone 16B12)	Primary	Mouse	Biolegend	901515	1:500 (WB)
HA-Tag	Primary	Rabbit	Cell Signaling Technologies	37245	1:500 (ICC)
Hdac2	Primary	Rabbit	Protein Tech Group	12922-3-AP	1:500 (WB)
Kars1	Primary	Rabbit	Protein Tech Group	14951-1-AP	1:500 (ICC, PLA)
Map2	Primary	Chicken	Abcam	ab5392	1:5000 (ICC)
Map2	Primary	Chicken	Novus	NB-300-213	1:500 (IHC)
Matr3	Primary	Rabbit	Protein Tech Group	12202-2-AP	1:100 (ICC, PLA)
Prox1	Primary	Rabbit	Protein Tech Group	11067-2-AP	1:2000 (WB)
Psd95	Primary	Mouse	Synaptic Systems	124011	1:500 (ICC)
PSD95	Primary	Mouse	NeuroMab	75-028	
Ranbp2	Primary	Rabbit	Bethyl Laboratories	A301-796A-T	1:1000 (WB)
Rangap1	Primary	Rabbit	Protein Tech Group	27405-1-AP	1:2000 (WB), 1:250 (ICC, PLA)
Shank2	Primary	Guinea Pig	Synaptic Systems	162204	1:2000 (IHC)
Sumo1	Primary	Mouse	Developmental Studies Hybridoma Bank	SUMO1 76-86	1:50 (WB)
Sumo2/3	Primary	Rabbit	Invitrogen	PA5-109851	1:500 (WB)
Sumo2/3 [8A2]	Primary	Mouse	Abcam	ab81371	1:500 (ICC, PLA)
Synapsin1	Primary	Mouse	Synaptic Systems	406011	1:1000 (ICC), 1:2000 (IHC)
Synaptophysin	Primary	Mouse	Synaptic Systems	101011	1:2000 (WB)
Trim28	Primary	Mouse	Abcam	22553	1:2500 (WB)
vgat1	Primary	Guinea Pig	Synaptic Systems	131004	1:1000 (IHC)
vGlut1	Primary	Mouse	Synaptic Systems	135511	1:1000 (IHC)
Secondary Antibody	Type	Host Species	Company	Catalog Number	Concentration
Anti-chicken AF633	Secondary	Goat	ThermoFisher	A21103	1:2000
Anti-chicken AF647	Secondary	Goat	ThermoFisher	A21449	1:1000
Anti-goat AF555	Secondary	Donkey	ThermoFisher	A21432	1:2000
Anti-guinea pig AF488	Secondary	Goat	ThermoFisher	A11073	1:2000
Anti-mouse AF488	Secondary	Goat	ThermoFisher	A11001	1:1000-1:2000
Anti-mouse HRP	Secondary	Goat	Biorad	172-1011	1:2000
Anti-mouse HRP	Secondary	Donkey	Jackson ImmunoResearch	711-035-150	1:10000
Anti-mouse HRP Light Chain Specific	Secondary	Goat	Jackson ImmunoResearch	115-035-174	1:10000
Anti-rabbit AF594	Secondary	Goat	ThermoFisher	A11012	1:1000
Anti-rabbit HRP	Secondary	Donkey	Jackson ImmunoResearch	711-035-152	1:10000
Anti-rabbit HRP Light Chain Specific	Secondary	Mouse	Jackson ImmunoResearch	211-032-171	1:10000
Duolink in-situ PLA probe an	Secondary	Donkey	Sigma-Aldrich	DUO92004	As Per Kit
Duolink in-situ PLA probe an	Secondary	Donkey	Sigma-Aldrich	DUO92002	As Per Kit

Acronym	Description
ICC	Immunocytochemistry (neuron culture)
PLA	Proximity Ligation Assay
IHC	Immunohistochemistry (brain tissue)
WB	Western Blot
IP	Immunoprecipitation

Chapter 4: General discussion and conclusions

4.1 Summary

ALS and FTD, although seemingly unique neurodegenerative diseases, exist on a clinical and genetic spectrum(Strong et al., 2017). While different cell types and brain regions can be affected across the disease, manifesting in highly heterogenous symptom presentation, TDP-43 pathology is pathognomonic for nearly all cases of ALS and almost half of FTD(Kawakami et al., 2019; Kwong et al., 2007). Over 50 mutations in TDP-43 are characterized to cause ALS/FTD (Ghasemi and Brown, 2018; Suzuki et al., 2022). However, most cases occur sporadically yet still present with TDP-43 pathology indicating that exogenous mechanisms converge on TDP-43 in disease. Prolonged cellular stress through environmental exposures or disruption of the cellular stress response – as observed in ALS/FTD patients – can result in the mislocalization and accumulation of TDP-43(Odierna et al., 2024; Ratti et al., 2020). However, the molecular mechanisms that directly regulate TDP-43 localization and metabolism upon stress remain elusive.

We found that in response to cellular stress, TDP-43 is SUMOylated within the nucleus upstream of aggregation, and that this phenomenon is proportional to the relative degree of stress experienced within the cell. Furthermore, we posited that TDP-43 SUMOylation is mediated through E3 SUMO ligases to facilitate stress-dependent SUMOylation enabling a high level of temporal control. Indeed, we identified 4 candidate E3 SUMO ligases, EGR2, PIAS1, TRIM28, and ZNF451 which may serve as effective targets to modulate levels TDP-43 SUMOylation. During stress recovery, TDP-43 SUMOylation continues before being cleared through the ubiquitin proteasome system. This suggests that SUMOylation may play key roles in regulating TDP-43 proteostasis during cellular stress and recovery.

Previous studies have suggested that TDP-43 becomes SUMOylated by SUMO1 at K136 (Maraschi et al., 2021). However, we mapped SUMOylation by SUMO2/3 to occur on K408 within the CTD, directly adjacent to phosphorylation sites characteristic of TDP-43 aggregates. Preventing phosphorylation by mutating S403/404/409/410 to alanine (A), led to increased TDP-43 SUMOylation suggesting the modifications may act antagonistically. We further found that these modifications reside within a highly conserved region of the CTD, suggesting that post translational modifications within this domain may be an important mode of regulation in vertebrates. To better understand the physiological roles of TDP-43 SUMOylation at K408, we generated a knock-in mouse model with a point mutation at K408 (“TDP-43^{K408R} mice”) to prevent endogenous TDP-43 SUMOylation. In primary neuron cultures from the TDP-43^{K408R} mice, we observed impairments in recovery from stress including delayed stress granule disassembly, excess formation of nuclear TDP-43 foci, and accumulation of insoluble phosphorylated TDP-43.

As TDP-43 SUMOylation is induced in response to stress, we hypothesized that aging would function as a stressor upon TDP-43 and cause age-dependent phenotypes to develop in TDP-43^{K408R} mice. Interestingly, we observed sex-specific and age-dependent phenotypes occurring as early as 2 months of age. Female mice presented with cognitive abnormalities in the open field, beam break, and y-maze tests in addition to changes in social behaviour observed in the tube-test of social dominance leading to significant barbering of mutant mice. Male mice did not show any phenotypic changes in behaviour and neither males nor females presented with motor impairment. However, on the histopathological and biochemical levels, we observed accumulation of insoluble phosphorylated TDP-43 in the cortex of female mice and in the spinal cords of both male and female mice. Furthermore, we observed significant TDP-43 mislocalization in motor neurons of male and female mice. At 16 months of age, we observed significantly fewer motor

neurons and denervation at neuromuscular junctions. Thus, male and female mice present with some early pathology related to ALS/FTD inferring that SUMOylation is a protective mechanism maintaining neuron health during aging.

We questioned whether SUMOylation may be a global regulatory mechanism that is important during human aging and found increased SUMOylation in temporal lobe samples of aged (>60-years-old) individuals without neurological abnormalities. When assessing TDP-43 SUMOylation in ALS/FTD cases against age and sex matched controls, we found that TDP-43 SUMOylation was significantly increased. Thus, there may be activation or increased dependency on the TDP-43 SUMOylation pathways in patients diagnosed with ALS/FTD.

Changes in global SUMOylation in the human brain across aging raises many questions about how SUMOylation and its specific targets play roles in health and disease. However, due to the highly dynamic nature of SUMOylation and high levels of homology between SUMO paralogs, unbiasedly identifying roles and targets of SUMOylation in tissue is considerably challenging. Thus, we generated a His₆-HA-SUMO2 knock-in mouse to complement a previously established His₆-HA-SUMO1 model (Tirard et al., 2012) expanding the tools available to facilitate direct comparison between SUMO1 and SUMO2 *in vivo*.

Using the His₆-HA SUMO1 and His₆-HA-SUMO2 models, we directly compared SUMO1 versus SUMO2 across different tissues *in vivo*. Using whole brain imaging, we generated an atlas of SUMO1 and SUMO2 distribution throughout the mouse brain. We found that SUMO2 levels differ across brain regions more than SUMO1 which may provide insight into key neural functions regulated by SUMOylation. We further demonstrated that SUMO2 natively localizes to synapse *in vivo*, despite generally thought of as a nuclear modification. Finally, using immunoprecipitation coupled to mass spectrometry, we demonstrated that these models enable the identification of

native SUMO1 and SUMO2 substrates from tissue and compared the selectivity of SUMOylation by each paralog. Together, these models will enable the exploration of SUMOylation throughout the whole mouse to better understand the roles of SUMOylation and each SUMO paralog in mammalian biology.

4.2 Discussion of Key Findings

TDP-43 is SUMOylated Selectively in Response to Cellular Stressors

Post translational modifications (PTMs) play key roles in regulating protein functions. Increasingly, TDP-43 is found to be modified by a variety of different PTMs enabling TDP-43 to perform unique roles and function in different pathways in a highly regulated manner (Buratti, 2018). Here we described TDP-43 as a target of SUMOylation, specifically by SUMO2/3, in response to select stressors. We demonstrated that sodium arsenite treatment and heat shock at 42 °C induce TDP-43 SUMOylation. However, treatment with MG132, D-Sorbitol, nor NaCl at concentrations reported to induce the formation of stress granules and affect TDP-43 have no effect on TDP-43 SUMOylation (Dewey et al., 2011; Gao et al., 2022; Hans et al., 2020; Mazroui et al., 2007). Thus, TDP-43 SUMOylation is contextual to specific insults. Similar findings have been observed for other stress related proteins such as stress-specific ubiquitination of G3BP1 (Gwon et al., 2021; Maxwell et al., 2021). Collectively, these findings highlight the complexity of cell stress responses.

Currently, the underlying commonalities between stressors that induce TDP-43 SUMOylation compared to those that do not remain unclear. Heat shock, sodium arsenite, and D-Sorbitol are all characterized to activate the ISR leading to phosphorylation of eIF2 α and activation of stress granules (Costa-Mattioli and Walter, 2020; Klein et al., 2022; Lee et al., 2021;

Pakos-Zebrucka et al., 2016). Furthermore, these stressors can induce mislocalization and aggregation of TDP-43 (Dewey et al., 2011; Hans et al., 2020; Udan-Johns et al., 2014). Thus, TDP-43 SUMOylation may not be directly downstream of eIF2a activation or stress granule formation nor do stresses that induce TDP-43 aggregation trigger SUMOylation. Sodium arsenite and heat shock can function as oxidative stressors and lead to proteotoxicity, however MG132 treatment does not induce TDP-43 SUMOylation (Bernstam and Nriagu, 2000; Mazroui et al., 2007; Slimen et al., 2014). Thus, unfolded protein responses may not be the commonality amongst these stressors. Better understanding the similarities and differences between pathways reactive to various stressors will be required to parse out how SUMOylation may play a role in response to certain stressors. Alternatively, our study selected concentration of stressors based on previous literature to induce TDP-43 aggregation and/or stress granule formation. It is not clear whether these stressors, although leading to the same pre-defined outcomes, induce the same burden of stress on cells. As we found that TDP-43 SUMOylation is responsive to relative stress burden, stressors that did not induce TDP-43 SUMOylation may not have achieved the threshold to induce this effect. Ultimately, TDP-43 SUMOylation may serve as a unique molecular cascade to help better understand how various stressors trigger different cascades within cells. Furthermore, better understanding the overlap and differences between stress pathways will uncover how some stressors may converge on TDP-43, potentially making them a risk for TDP-43 dysfunction and disease.

Nuclear regulation of TDP-43 upstream of pathology

The chronology of TDP-43 pathology is critically important to understand to parse out direct drivers of neurodegeneration and late-stage consequences of neurotoxicity. This knowledge

will help identify key biomarkers of disease risk and progression and potential preventative therapeutic targets. Late-stage TDP-43 pathology has garnered much attention due its widespread prevalence in ALS/FTD patients and the striking cellular phenotype surrounding TDP-43 nuclear depletion and its cytoplasmic aggregation(Hayes and Kalab, 2022; Scotter et al., 2015). However, increasing evidence suggests that the formation of TDP-43 aggregates is a late-stage event in neurodegeneration and that nuclear to cytoplasmic mislocalization occurs upstream of aggregation(Baughn et al., 2023; Brown et al., 2022; Coyne et al., 2021; Klim et al., 2019; Krus et al., 2022; Ma et al., 2022; Melamed et al., 2019; Suk and Rousseaux, 2020). Indeed, recent studies have identified key events such as cryptic splicing that occurs in disease due to TDP-43 loss of function from the nucleus. These events not only serve as risk factors for disease, such as cryptic mis-splicing of *UNC13A* – mutations in which elevate one’s risk for ALS/FTD but can also drive neurodegeneration as exemplified through cryptic mis-splicing of *STMN2*(Brown et al., 2022; Klim et al., 2019; Ma et al., 2022; Melamed et al., 2019). Cryptic mis-splicing events can even serve as biomarkers of TDP-43 loss of function. For example, upon TDP-43 loss of function, inclusion of a cryptic exon in *HDGFL2* leads to an in-frame peptide insertion(Seddighi et al., 2024). This peptide can be targeted by antibodies as a fluid biomarker of TDP-43 dysfunction(Calliari et al., 2024). Although loss of nuclear function is typically associated with TDP-43 mislocalization, further nuclear dysfunction can also occur during TDP-43 pathogenesis. Recent studies using RNA aptamers to visualize TDP-43 observed nuclear pathology that correlated with cryptic mis-splicing and preceded ALS symptoms(Spence et al., 2024).

Our findings demonstrate that TDP-43 is SUMOylated and polySUMOylated in the nucleus rapidly in response to stress upstream of aggregation. Upon removal of stress, SUMOylated TDP-43 is then cleared through the Ubiquitin Proteosome System (UPS). We further

found that blocking TDP-43 SUMOylation alters the stress response and leads to TDP-43 and ALS/FTD-like pathology *in vivo*. We hypothesize that blocking TDP-43 SUMOylation prevents clearance through the UPS pathway, thus activating alternative routes of clearance such as mislocalization to take advantage of proteostasis mechanisms outside of the nucleus. Upon aging, many of these proteostasis mechanisms characteristically become less effective, which may lead to TDP-43 accumulation and pathology. Thus, dysfunction of TDP-43 SUMOylation could be a risk factor for ALS/FTD.

Determining the state of TDP-43 SUMOylation in disease remains challenging due to limitations of available tools. For example, we employed PLA in human patient tissue and observed significantly increased interactions between TDP-43 and SUMOylation. However, PLA is not effective at identifying SUMOylation at specific sites thus the data may be interpreted as increased interactions between TDP-43 and SUMO occur in disease. This is supported by evidence suggesting that insoluble TDP-43 aggregates can be enriched for SUMO2/3(Seyfried et al., 2010). Using novel techniques such as RNA aptamers designed to recognize site-specific SUMOylation will help facilitate how SUMOylation at K408 is linked to ALS/FTD. Additionally, genetics studies associating genes related to SUMOylation and ALS/FTD can further our understanding of how SUMOylation pathways may be disrupted in disease. Interestingly, haploinsufficiency-inducing variants in the *SUMO4* gene have been associated with ALS risk, potentially via an impaired cellular stress response(Osmanovic et al., 2022). SUMO4 is most closely related to SUMO2, however not much is known about its function but is also thought to be stress responsive(Baczyk et al., 2017; Bouchard et al., 2021; Citro and Chiocca, 2013). As increasing whole genome sequencing studies are performed, deep analysis of SUMOylation-related genes may help uncover how SUMOylation may be implemented in disease.

Post translational regulation at the TDP-43 C-terminal domain

The C-terminal domain (CTD) is a particularly critical region of TDP-43 for its involvement in ALS/FTD (Berning and Walker, 2019; Igaz et al., 2009; Kasu et al., 2018; Walker et al., 2015). Not only are most ALS/FTD causing mutations found within the CTD, but the intrinsically disordered structure is thought to be a key factor in the propensity for TDP-43 to aggregate (Gendron et al., 2012; Ticozzi et al., 2011). Thus, understanding the direct functions and regulation of the CTD can give insight into TDP-43 pathogenesis in neurodegeneration.

When analyzing the C-terminus of TDP-43 containing the phosphorylation and SUMOylation sites, we found that the extreme C-terminus was highly conserved amongst vertebrate species, whereas the remainder of the CTD is less conserved. The SUMOylation motif surrounding (human) K408 is also particularly well conserved, considering the Aspartic Acid (D) D406 evolved from a Glutamic Acid (E) at the same residue. This would suggest the consensus SUMOylation sequence (KxD/E) was maintained with the amino acid evolution that occurred between amphibians and humans (Rodriguez et al., 2001). The co-evolution and occurrence of the spinal cord and the TDP-43 C-terminal domain could suggest this domain, and its regulation, are important for spinal cord health. Our findings demonstrate that SUMOylation at K408 plays a role in regulating TDP-43 proteostasis during cellular stress and recovery. In adjacent fields studying C-degrons – a mechanism that regulates protein degradation through the C-terminus – various proteins are characterized to contain conserved C-terminus regions for the purpose of maintaining proteostasis (Varshavsky, 2019; Yeh et al., 2021). This mechanism is also characterized to help degrade proteins when they become mislocalized to the cytoplasm (Yeh et al., 2021). However, whether C-degrons act upon TDP-43 has not been studied. It is suggested that proteins have

evolved more complex systems at the C-terminus to protect against C-degron degeneration and provide more fidelity in protein regulation and degradation(Yeh et al., 2021). Our findings with respect to SUMOylation at K408 align with the notion that the C-terminus of TDP-43 is involved in proteostasis. However, the extent of which, and the level of regulation available through the regulation by multiple PTMs requires further exploration.

Phosphorylation of the CTD is a hallmark of TDP-43 aggregates and is also highly conserved alongside SUMOylation(Neumann et al., 2009a). However, the physiological functions of phosphorylation remain unclear. Some studies have suggested that phosphorylation of the CTD mediates phase transition dynamics of TDP-43 by altering biophysical properties within the condensates (Haider et al., 2024). Recent studies suggest that phosphorylation at S403/404 may occur upstream of phosphorylation at S409/410, potentially in response to stress followed by late state aggregation, respectively(De Rossi et al., 2021). Phosphorylation of some proteins can enable SUMOylation, particularly when containing a phosphorylation-dependent SUMOylation motif (PDSM). These PDSMs have not been extensively characterized, but many of the known proteins containing a PDSM function as transcription factors(Hietakangas et al., 2006). Surprisingly, we found that blocking phosphorylation did not influence SUMOylation but led to increased SUMOylation. This may suggest that phosphorylation and SUMOylation function antagonistically and that phosphorylation may function to block or remove SUMOylation from TDP-43. For example, phosphorylation at the residues adjacent to K408 may alter the structure or charge of the SUMOylation motif sufficiently to prevent the interaction with UBC9 and E3 SUMO ligases thereby preventing SUMOylation. Conversely, TDP-43 SUMOylation at K408 may provide steric hindrance and prevent phosphorylation at adjacent residues. These scenarios may suggest that phosphorylation and SUMOylation confer unique functions upon TDP-43. Alternatively,

phosphorylation may signal for deSUMOylation to occur, thereby altering the fate and function of TDP-43. Taken together, phosphorylation and SUMOylation can confer complex regulation of TDP-43. Future work should aim better understand the interplay between the modifications and how they may play unique or complementary regulatory roles.

TDP-43^{K408R} mice as a potential mouse model of sporadic ALS/FTD

Modeling ALS and FTD, particularly in mouse models, is challenging to recapitulate key phenotypes associated with disease. Mouse models expressing mutant SOD1 are capable of recapitulating key aspects of ALS such as motor neuron degeneration, progressive muscle paralysis and motor impairment, and early lethality(Bruijn et al., 1997). However, SOD1 ALS only represents a small fraction of all ALS cases and are generally linked with fALS(Nguyen et al., 2018). As TDP-43 pathology is associated with nearly all cases of ALS and half of FTD, the development of a TDP-43 centered model is critical to model disease representative of most ALS cases(De Boer et al., 2021; Kwong et al., 2007). To this end, models overexpressing TDP-43 present with highly aggressive phenotypes. For example, the human TDP-43 overexpression mouse model causes motor impairment, paralysis, and early lethality by P30(Wils et al., 2010). However, overexpression models do not best represent the natural progression of disease and lead to transcriptomic changes that do not align with ALS/FTD(Carmen-Orozco et al., 2024).

In contrast, knock-in mouse models affecting endogenous TDP-43 are generally quite mild, presenting with some aspects of disease, but rarely recapitulate the constellation of phenotypes associated with ALS/FTD. For example, knock-in of the ALS/FTD causing p.Q331K mutation into mouse TDP-43 does not lead to motor impairment or signs of TDP-43 pathology(White et al., 2018). There are however a few mild cognitive impairments and changes in the transcriptome that

relate to ALS/FTD. Several other knock-in mouse models have been generated using ALS causative mutations such as p.A315T, however they generally fail to recapitulate many of the aspects of ALS/FTD(S. L. Huang et al., 2020). Endogenous knock-in of p.N390D into TDP-43 does show some key aspects of ALS such as early lethality, neuromuscular junction denervation and muscle atrophy, and TDP-43 mislocalization and pathology. However, these phenotypes take 6 to 24 months to develop and represent specific ALS-linked mutations.

Although the TDP-43 knock-in models may represent some aspects of ALS, they rely on ALS/FTD causing mutations to drive phenotypes. Most cases of ALS are not driven by TDP-43 mutations, thus models that act upon TDP-43 and can recapitulate TDP-43 pathology and disease phenotypes are critical to represent the majority of ALS/FTD patients(Kirola et al., 2022; Nguyen et al., 2018). Recently, a mouse model replicating acetylation of TDP-43 through a p.K145Q acetyl-mimetic mutation was generated leading to loss of RNA binding and development of cognitive impairment potentially modeling aspects of sporadic FTD(J. Necarsulmer et al., 2023). Our TDP-43 mouse model bearing an endogenous p.K408R mutation to block endogenous SUMOylation also lead to some cognitive impairments. However, we also observe significant neuromuscular junction denervation and TDP-43 mislocalization in the spinal motor neurons thus suggesting that blocking TDP-43 SUMOylation can lead to early features of both ALS and FTD. A major consideration for the TDP-43^{K408R} mouse model is that we hypothesize phenotypes will be dependent on stress, thus applying relevant stressors to the mice beyond aging may lead to more severe phenotypes better modeling sporadic ALS/FTD mediated through environmental interactions.

Sex-specific impairment in TDP-43^{K408R} mice.

A surprising finding in the TDP-43^{K408R} mouse model is the development of sex-specific impairment. ALS carries a slight male bias and FTD is generally considered to affect sexes equally, but this may be more complicated when focusing on specific subtypes of FTD (Curtis et al., 2017). Nevertheless, we expected that potential sex differences would be rather mild. However, we observed several cognitive changes specifically in female mice by blocking endogenous TDP-43 SUMOylation. These cognitive changes relate to social behaviour, as observed by increased barbering and submissiveness, changes in overall activity and risk taking, and memory deficits. None of these phenotypes were observed in male mice. While some of these phenotypes correlate with aspects of FTD, they may also highlight unexpected processes regulated by TDP-43 SUMOylation in a sex-specific manner warranting further investigation. Additionally, we observed significant degeneration of motor neurons and corresponding neuromuscular junction denervation in aged male mice. This may reflect sex specific roles for TDP-43 SUMOylation in the spinal cords of male mice or may be a result of potentially delayed degeneration in females that was missed at the 16-month timepoint when analysis occurred. In the latter scenario, this could also represent a sex specific effect that may act upon males differently than females. For example, motor neurons in male mice may experience more stress than female mice due to increase body and muscle mass leading to early stress-dependent phenotypes (Odierna et al., 2024; Van Den Bosch et al., 2006; Yamashita et al., 2023). Alternatively, several studies have uncovered protective roles for estrogen in neurodegenerative diseases which may delay the onset of phenotypes in female mice (Handley et al., 2022; Isojärvi, 2003; Yamashita et al., 2023). Future work will have to carefully parse out sex effects that are native to male and female biology, such as gene/protein expression and hormones, from sex effects that are related to natural behavioural and biological differences that

can feed back on the TDP-43 SUMOylation pathway. These analyses will help to better understand how both TDP-43 and SUMOylation is differentially involved in biology relating to sex differences in the CNS.

SUMOylation as a protective mechanism in neuronal aging

Although SUMOylation is a highly conserved and essential mechanism, we are only beginning to understand the specific processes regulated by SUMOylation, particularly within the CNS (Celen and Sahin, 2020; Geiss-Friedlander and Melchior, 2007). SUMOylation is essential for mammalian CNS development and has been linked to important developmental processes such as neurogenesis (Correa-Vázquez et al., 2021; García-Gutiérrez and García-Domínguez, 2022; Hasegawa et al., 2014; Talamillo et al., 2020; Wang et al., 2014; Yau et al., 2020; Zhang et al., 2008). Global SUMOylation fluctuates during developmental stages leading up to adulthood in murine models, however it is unclear how SUMOylation is related to aging (Tirard et al., 2012). We found that global SUMOylation via SUMO2/3 is upregulated in elderly individuals unaffected by neurodegenerative or neurological diseases. Although it is unclear the cause of this global shift it is likely linked to key processes that characteristically dysfunction as a part of natural aging. These processes include changes in proteostasis, particularly with regards to the UPS and autophagy pathways, as well as accumulation of DNA damage and increase ROS production (López-Otín et al., 2013; Maragkakis et al., 2023; Vijayakumaran and Pountney, 2018). Each of these processes are highly linked with SUMOylation and stress responses, thus SUMOylation may be responding to these naturally occurring stresses that accumulate during aging. Future work uncovering specific targets of SUMOylation that are upregulated in aging will help to understand key pathways that are activated or disrupted in healthy human aging.

Understanding these healthy pathways will be critical to understand the complex changes that can occur in age-related neurodegenerative diseases.

Direct comparisons of SUMO1 and SUMO2 in vivo.

Comparison of SUMO paralogs *in vivo* have remained highly challenging due to the dynamic nature of SUMOylation and the poor specificity of antibodies against specific SUMO paralogs(Alexander J. Garvin et al., 2022). Therefore, there is a reliance on overexpression systems in cell models to study SUMOylation(Geiss-Friedlander and Melchior, 2007). This can lead to artifacts and may not reflect the *in vivo* state. Studies have found that most antibodies against SUMO paralogs have major drawbacks regarding specificity or sensitivity, and rarely show consistency across various assays(Alexander J. Garvin et al., 2022). Studies of specific targets of SUMO paralogs have drawn controversy as to the validity of findings from cell models and overexpression systems and how well they represent native protein regulation *in vivo*(Daniel et al., 2018; Henley et al., 2021; Martin et al., 2007; Matsuzaki et al., 2015; Tirard et al., 2012). The His₆-HA-SUMO mice allow for the use of highly validated anti-HA antibodies to directly compare SUMO paralogs. This importantly enables direct comparisons of SUMO1 and SUMO2 without artifacts mediated using different antibodies with suboptimal efficacies. Furthermore, these models provide the ability to study SUMOylation in different tissues and physiological states and take advantage of the tools designed to study proteins using HA epitopes.

4.3 Future Directions

Uncovering the molecular roles for TDP-43 SUMOylation

While we demonstrated that TDP-43 becomes SUMOylated in response to stress then cleared through the UPS pathway during recovery, it remains unclear why SUMOylation occurs in the first place if to be degraded by the UPS. This may suggest that SUMOylation has an intermediate role upstream of UPS degradation. For example, SUMOylation and polySUMOylation plays roles in maintaining solubility and phase separation or regulating DNA interactions(Cheng, 2023; Keiten-Schmitz et al., 2021). Therefore, in response to stress TDP-43 may become SUMOylated to perform these functions and then be cleared during recovery. However, determining the exact function of TDP-43 SUMOylation at K408 remains challenging due to the low levels of SUMOylated TDP-43 and the lack of available tools to study TDP-43 in its SUMOylated state.

Post translational modifications such as phosphorylation or acetylation, often phospho- or acetyl- mimetic mutations such as Aspartic Acid (D)/Glutamic Acid (E) or Glutamine (Q), respectively, mimic constitutive modifications(Allen et al., 2024; Wang and Hayes, 2008). Due to the large size of the SUMO modification, mimetics are not possible. Some studies have generated fusion proteins to mimic SUMO bound to the protein, but these are artificially introduced into cells through transgenes and function as a constitutive SUMO-protein fusion and therefore do not represent site-specific modification(Zhu et al., 2023). Thus, the primary method to study the effects of SUMOylation is through loss of function by blocking the SUMOylation site(Impens et al., 2014). This is further complicated for TDP-43 as SUMOylation only occurs in a stress dependent manner and only upon a fraction of all TDP-43.

Unbiased studies can enable global analyses into functions that are altered upon loss of TDP-43 SUMOylation. For example, using proximity labeling methods such as BioID or TurboID to compare interactomes of TDP-43 and TDP-43^{K408R} in unstressed, stressed, and recovery conditions may help to identify specific complexes associated with SUMOylated TDP-43 transiently during stress (i.e. interactions that are lost upon p.K408R mutation)(Cho et al., 2020; Roux et al., 2012; Sears et al., 2019). Similarly, RNA sequencing approaches may provide insight into global pathways that are altered upon loss of TDP-43 SUMOylation, inferring potential roles for SUMOylated TDP-43. Identifying these pathways and interactors will not only uncover potential genes/proteins associated with neurodegeneration, thus further linking TDP-43 SUMOylation with disease, but may also identify inroads to restore cellular function upon loss of TDP-43 SUMOylation.

TDP-43^{K408R} mice as a “sensitized” model of TDP-43 pathology

As TDP-43 SUMOylation is dependent upon cellular stress, we initially posited that aging would function as a stressor to induce TDP-43 SUMOylation via various mild insults throughout life. We hypothesized that blocking TDP-43 SUMOylation in the TDP-43^{K408R} mouse model would lead to accumulation of TDP-43 in an age-dependent manner leading to characteristics of ALS/FTD. Indeed, we did observe some phenotypes resembling aspects of ALS/FTD pathology including TDP-43 accumulation, cognitive impairment in female mice, and motor neuron disruption in male mice. However, these phenotypes remained relatively modest and did not encompass the constellation of phenotypes observed in ALS/FTD. Therefore, we posit that blocking TDP-43 SUMOylation may confer a risk for TDP-43 proteinopathies.

As mouse aging in a laboratory vivarium does not mimic aging that occurs in humans throughout life, we hypothesize that additional extrinsic insults can push TDP-43 to trigger disease-like phenotypes (Dutta and Sengupta, 2015; Zhuang et al., 2019). The TDP-43^{K408R} mouse model is uniquely poised to test how ALS/FTD relevant stressors may exacerbate TDP-43 pathology leading to better mouse models of TDP-43 pathology and further our understanding of the protective functions of SUMOylation. Key stressors such as environmental toxins, virus infections, fevers (heat shock), trauma, or genetic susceptibility can all induce various forms of stress throughout an individual's lifetime and have been linked to ALS/FTD (Yu and Pamphlett, 2017; Yu et al., 2014). For example, *C9ORF72* HRE mouse models display modest phenotypes related to TDP-43 pathology and ALS/FTD-like behavioural impairment, however, do not faithfully recapitulate key features of ALS/FTD (Chew et al., 2019, 2015; Cook et al., 2020; Herranz-Martin et al., 2017). Crossing *C9ORF72* HRE mice to the “stress sensitive” TDP-43^{K408R} model would likely trigger stress responses that depend on TDP-43 SUMOylation. The combination of partially penetrant *C9ORF72* HRE along with stress sensitive TDP-43 would likely induce TDP-43 pathology more rapidly as SUMOylation is unable to clear TDP-43 through the UPS. Ultimately, determining how external insults converge on TDP-43 to induce pathology is key to modeling sporadic forms of ALS.

Beyond genetic insults, heat shock mouse models can induce TDP-43 pathology and phenotypes related to ALS/FTD (Dubinski et al., 2023). Applying this stress to the TDP-43^{K408R} mice will likely worsen the preexisting phenotypes pushing TDP-43 toxicity to better reflect disease states. If this is the case, these combinatorial models would also provide the opportunity to determine whether SUMOylation may be a viable therapeutic target. This can be achieved through overexpression of the E3 ligases to enhance TDP-43 SUMOylation. We would expect that

pathology caused by heat shock exposure would be less severe upon overexpression of the E3 SUMO ligase targeting TDP-43 thereby promoting effecting recovery from stress.

Together, the TDP-43^{K408R} mouse model may serve as the foundation to study TDP-43 pathology without dependence on disease causing mutations. This will enable a shift to better understand and model sporadic forms of ALS thereby uncovering the causes of disease for the majority of ALS/FTD cases.

Identifying novel SUMO substrates in the brain related to aging and neurodegeneration

We demonstrated that TDP-43 SUMOylation is a protective mechanism preserving neuronal function. However, many proteins are natively SUMOylated in the brain and likely play critical roles in neuron longevity alongside or independent of TDP-43(Lumpkin et al., 2017; Pronot et al., 2021; Tirard et al., 2012). Additionally, we observed that global SUMOylation changes over time and is positively correlated with aging. Identifying other proteins that are associated with aging and neurodegeneration will provide new insight into protective and toxic mechanisms in disease. The His₆-HA-SUMO1 and His₆-HA-SUMO2 mouse models serve as valuable tools to study native SUMOylation in the mammalian brain(Suk et al., 2023; Tirard et al., 2012).

Aging studies coupled with immunoprecipitation and mass spectrometry (IP/MS) from brains of His₆-HA-SUMO1 and His₆-HA-SUMO2 mouse models can help to identify novel and disrupted targets of SUMOylation during mammalian aging. These targets will provide insight into key pathways that are altered during aging and may help to identify proteins associated with age-related neurological diseases. Beyond aging studies, activity-dependent SUMO proteomes can also be studied. Previous reports have identified global changes in SUMOylation in the hippocampus of mice that were subject to fear conditioning behaviour tests which involve an electric

shock(Gustin et al., 2022). This fear response induced global SUMOylation in the hippocampus. Using the His₆-HA-SUMO models, applying a stimulus such as a shock then coupling IP/MS can help identify novel SUMOylated proteins involved in fear responses. Relating to neurodegenerative disease, heat shock of mice leads to induction of stress responses and pathology related to ALS/FTD(Dubinski et al., 2023). Identifying SUMOylated proteins in response to heat shock can help identify key pathways involved in the native neuronal stress response, such as TDP-43.

Finally, the His₆-HA-SUMO1 and His₆-HA-SUMO2 mouse models can be combined with other mouse models to study more complex phenomena and the involvement of SUMOylation. For example, crossing the His₆-HA-SUMO lines to disease models such as the Tdp-43 p.Q331K model can help to identify SUMOylated proteins changes in mice affected by TDP-43 mutations(White et al., 2018). These may help to identify novel targets whereby blocking or restoring SUMOylation may be therapeutically beneficial. Crossing His₆-HA-SUMO mice with fluorescent reporter lines can allow for highly specific analysis of SUMOylation *in vivo*. For example, a His₆-HA-SUMO crossed with a reporter line with a nuclear fluorescent protein can allow isolation of cell-type specific nuclei through flow cytometry followed by IP/MS to identify native cell type specific substrates of SUMOylation. Taken together the His₆-HA-SUMO will serve as a foundational model to help facilitate the substrate specific identification of native SUMO substrates and their roles in brain health and disease.

Appendix I

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Appendix II

Permission to reprint published manuscripts

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A stress-dependent TDP-43 SUMOylation program preserves neuronal function

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Characterizing the differential distribution and targets of Sumo1 and Sumo2 in the mouse brain

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Constitutive nuclear accumulation of endogenous alpha-synuclein in mice causes motor impairment and cortical dysfunction, independent of protein aggregation



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Van Bruggen, R. Patel, Z.H., Wang, M., Suk, T.R., Rousseaux, M.W.C., Tan, Q. A versatile strategy for specific genetic manipulation of Cajal-Retzius cells in the adult mouse hippocampus. (2023) – *eNeuro*.

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Research Article | Research Article: Methods/New Tools, Novel Tools and Methods

A Versatile Strategy for Genetic Manipulation of Cajal–Retzius Cells in the Adult Mouse Hippocampus

Rebekah van Bruggen, Zain H. Patel, Mi Wang, Terry R. Suk, Maxime W. C. Rousseaux, and Qiumin Tan
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In this issue



eNeuro
Vol. 10, Issue 10
October 2023
[Table of Contents](#)
[Index by author](#)
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Appendix III

Appended Articles

Suk, T.R. and Rousseaux, M.W.C. The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. (2020) – *Molecular Neurodegeneration* **15**.

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REVIEW

Open Access

The role of TDP-43 mislocalization in amyotrophic lateral sclerosis



Terry R. Suk^{1,2} and Maxime W. C. Rousseaux^{1,2,3,4*} 

Abstract

Since its discovery as a primary component in cytoplasmic aggregates in post-mortem tissue of patients with Amyotrophic Lateral Sclerosis (ALS), TAR DNA Binding Protein 43 kDa (TDP-43) has remained a central focus to understand the disease. TDP-43 links both familial and sporadic forms of ALS as mutations are causative for disease and cytoplasmic aggregates are a hallmark of nearly all cases, regardless of TDP-43 mutational status. Research has focused on the formation and consequences of cytosolic protein aggregates as drivers of ALS pathology through both gain- and loss-of-function mechanisms. Not only does aggregation sequester the normal function of TDP-43, but these aggregates also actively block normal cellular processes inevitably leading to cellular demise in a short time span. Although there may be some benefit to therapeutically targeting TDP-43 aggregation, this step may be too late in disease development to have substantial therapeutic benefit. However, TDP-43 pathology appears to be tightly linked with its mislocalization from the nucleus to the cytoplasm, making it difficult to decouple the consequences of nuclear-to-cytoplasmic mislocalization from protein aggregation. Studies focusing on the effects of TDP-43 mislocalization have demonstrated both gain- and loss-of-function consequences including altered splicing regulation, over responsiveness to cellular stressors, increases in DNA damage, and transcriptome-wide changes. Additionally, mutations in *TARDBP* confer a baseline increase in cytoplasmic TDP-43 thus suggesting that small changes in the subcellular localization of TDP-43 could in fact drive early pathology. In this review, we bring forth the theme of protein mislocalization as a key mechanism underlying ALS, by highlighting the importance of maintaining subcellular proteostasis along with the gain- and loss-of-functional consequences when TDP-43 localization is dysregulated. Additional research, focusing on early events in TDP-43 pathogenesis (i.e. to the protein mislocalization stage) will provide insight into disease mechanisms, therapeutic targets, and novel biomarkers for ALS.

Keywords: ALS, TDP-43, Mislocalization, Pathology, Nucleocytoplasmic shuttling

Background

TDP-43, a central player in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective loss of motor neurons resulting in mortality within an average of 2–5 years [1]. Though most cases of ALS are sporadic (sALS), approximately 10% are familial (fALS) in origin. The identification of these familial cases, now spanning

over 20 genes (reviewed by Nguyen et al. [2]) has highlighted the importance of various cellular processes in the pathogenesis of ALS [3]. Indeed, some rare genetic cases – such as the identification of mutations in *TAR DNA Binding Protein 43 kDa* (*TARDBP*, encoding TDP-43) have provided crucial insight into common pathogenic themes in ALS [4–7].

TDP-43 bridges the divide between sporadic and familial ALS and remains a dominant protein of interest to understand disease pathogenesis. TDP-43 was identified as a primary component of ubiquitinated and hyper-phosphorylated cytosolic aggregates

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observed from post-mortem tissue of patients with ALS [8, 9]. This pathological phenomena is considered a hallmark of ALS as it is observed in approximately 97% of all ALS patients regardless of the mechanisms of disease onset, with the notable exceptions of familial ALS (fALS) caused by mutations in *Zn/Cu Superoxide Dismutase 1* (*SOD1*) and *Fused in Sarcoma* (*FUS*) [1, 10–15]. Furthermore, since the first report in 2008, over 50 mutations in *TARDBP* have been linked to ALS, further supporting TDP-43 dysfunction as a critical component in ALS [4–6, 16–18]. Therefore, TDP-43 dysfunction provides common ground in an otherwise convoluted disease, thus gaining notoriety and attention from researchers aiming to uncover the mechanisms causing TDP-43 aggregation. It is also important to note that mutations in *TARDBP* can also cause frontotemporal lobar dementia (FTLD), which itself shares some clinical parallels with ALS and displays TDP-43 pathology in ~45% of cases [8, 9, 19–21]. Here, however, we will focus on TDP-43 dysfunction as a central mechanism connecting multiple pathways in the context of ALS.

Main text

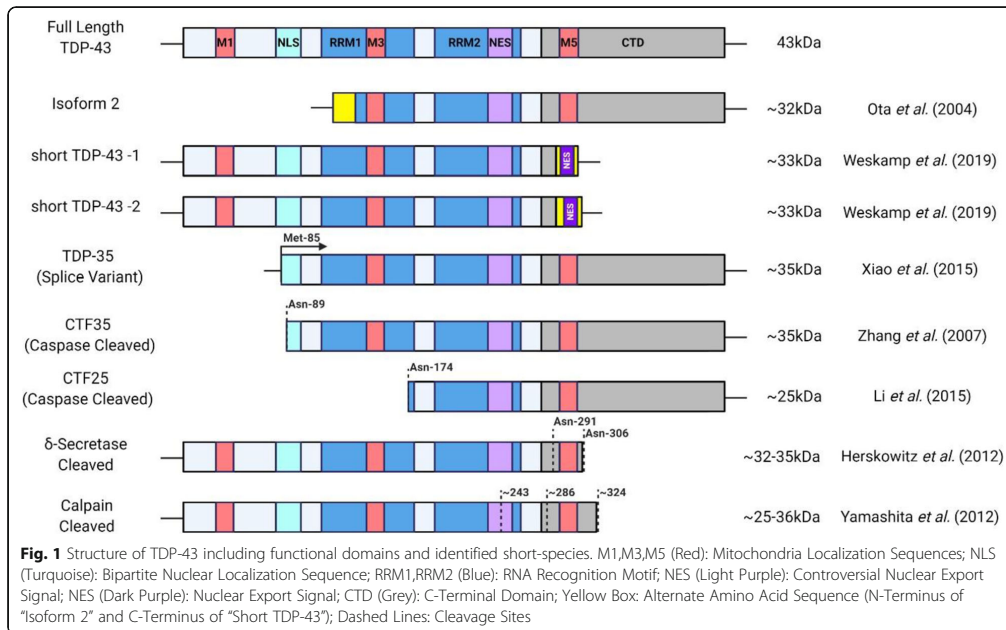
TDP-43 function, dysfunction, and aggregation

TDP-43 is a highly conserved and essential DNA/RNA binding protein belonging to the heterogeneous ribonucleoprotein family that preferentially recognizes UG-rich and TG-rich motifs of RNA and DNA, respectively [22–26]. TDP-43 is ubiquitously expressed in all cell types and is predominantly localized to the nucleus, but is also present in the cytoplasm and mitochondria [27–29]. Importantly, TDP-43 is highly regulated, particularly by autoregulation through cryptic exon repression within the 3'UTR of *TARDBP* mRNA [30–32]. Deletion of TDP-43 results in embryonically lethality in mice, and its depletion or overexpression causes toxicity or cell death in cell and animal models [33–48]. Structurally, TDP-43 has a bipartite NLS sequence in the N-terminal domain upstream of the first RNA recognition motif (RRM), a nuclear export signal (NES) within the second RRM, and 5 putative mitochondria localization signals (M1–M5) of which 3 (M1, M3, and M5) are functionally characterized [14, 24, 28, 29]. The NLS and NES are important for shuttling TDP-43 between the nucleus and cytoplasm, however the involvement of the NES remains controversial as some studies suggest the NES is non-functional [27, 49–51]. These motifs reside within the N-terminal portion of TDP-43 forming a globular tertiary structure [22, 52, 53]. The C-terminal domain (CTD) – sometimes referred to as the low-complexity domain (LCD), glycine-rich region, intrinsically disordered region (IDR), or prion-like domain (PrLD) – remains relatively unstructured and is thought to be critically important for TDP-43 toxicity in disease [4, 53, 54]. Not only is the unstructured nature of the CTD aggregation-prone, but

nearly all ALS-causing mutations on TDP-43 cluster within this domain [4, 6, 7].

In ALS, truncated forms of TDP-43 are found in ALS aggregates, more predominantly in the cortex but also to a lesser extent in the spinal cord [55–59]. The N-terminally truncated, C-terminal fragments 35 kDa (CTF35) and 25 kDa (CTF25) are the most notable “species” of TDP-43 [8, 60–62]. Several species of TDP-43 exist and are produced through translation of alternatively spliced isoforms or through proteolytic cleavage at the post-translational level (Fig. 1). CTF35 and CTF25 can be generated through proteolytic cleavage via Caspases 3 and 7 after asparagine-89, and Caspase 4 after asparagine-174, respectively, and caspase activity is also modulated by the ALS-linked protein Progranulin (PGRN) [63–69]. Alternative splicing also contributes to short forms of TDP-43 where a second splice isoform was identified through cDNA sequencing encoding an N-Terminally truncated, ~32 kDa isoform of TDP-43 [70]. Additionally, CTF35 fragment can also be generated through non-canonical splicing in exon 2 and alternative translational initiation at methionine-85 [59]. C-terminally truncated species can also be generated through proteolytic cleavage. δ -secretase cleaves TDP-43 after asparagine-291 and -306 to generate a ~32 kDa and ~35 kDa species, respectively [71]. The calcium-dependant cysteine proteases, calpains, also play a role in TDP-43 cleavage generating ~35 kDa and ~25 kDa species associated with cell toxicity [72, 73]. As many of the truncated species of TDP-43 are of similar molecular weights many studies simply nest them as “CTF35” or “CTF25” based on molecular weight without investigation to the exact species observed which may limit the understanding of TDP-43 species contribution to ALS as different species display distinctive properties [59, 63, 74, 75]. The exact functions of these truncated species remain unclear and are generally thought to be toxic, but have also been proposed to serve a protective role in the cell to promote TDP-43 clearance [59, 63, 73, 75–80]. It is important to recognize that other species of TDP-43 CTFs have been identified at 15–16 kDa, 22–25 kDa, and 33–37 kDa in ALS/ALS-FTLD, however due to low levels of reporting their prevalence in disease remains elusive [56, 74, 75, 81–85].

There are several features that commonly define aggregates of TDP-43 in ALS. These include the accumulation of post translational modifications such as ubiquitination, poly-ubiquitination, and aberrant phosphorylation (sometimes referred to as hyperphosphorylation) of full length TDP-43; specifically phosphorylation of TDP-43 at serine 409 and 410 (S409/410) is widely used as an indicator of aggregated TDP-43 [8, 9, 56, 58, 61]. TDP-43 aggregates in ALS also accumulate full length and lower molecular weight species of TDP-43 and stain positive for the ALS-



linked ubiquitin-binding autophagic adaptor Sequestosome 1 (SQSTM1, also known as p62) [8, 55–60, 82, 86–89].

The exact mechanisms mediating the formation of TDP-43 aggregates remain elusive. In ALS aggregates, TDP-43 was found to colocalize with important markers of stress granules (SGs) [90–95]. SGs are membraneless organelles that form in the cytoplasm comprised primarily of ribonuclear proteins and mRNA stalled in translation (Reviewed by Wolozin & Ivanov [96]). The formation of SGs occurs through a process called liquid-liquid phase separation (LLPS) where SG proteins and associated mRNA will de-mix into a liquid phase distinct from the cytosol [97, 98]. Two prominent proteins that are indicative of a SG are Ras GTPase-activating protein-binding protein 1 (G3BP1) and TIA1 cytotoxic granule-associated RNA binding protein (TIA1, [99–102]). Interestingly, mutations in the LCD of TIA1 – a domain that plays a key role in LLPS – cause ALS, further supporting the involvement of the cellular stress response in disease [103].

TDP-43 plays an important role in regulating the dynamics of SG formation and disassembly where loss of TDP-43 reduces SG formation [104, 105]. Treatment of cells with cell stressors used to study the formation of SGs, such as oxidative stressors (e.g. Sodium Arsenite), osmotic stressors (e.g. D-Sorbitol) or heat shock, results in the formation of phase-separated TDP-43 structures in the cytoplasm. Nevertheless, there remains a debate

in the field as to whether cytoplasmic TDP-43 structures indeed colocalize as a component of SGs or are mostly distinct from these bodies [90, 91, 93, 94, 103, 104, 106–109]. Under prolonged stress conditions, phase-separated TDP-43 transitions from a liquid-like droplet to form gel-like inclusions inhibiting their ability to dissociate [110–112]. These gel-like inclusions eventually accumulate several hallmarks the TDP-43 inclusions seen in ALS [61, 108, 109, 111].

Clearance of TDP-43 remains an important biological process tightly coupled with cytotoxicity. The ubiquitin-proteasome system is disrupted by ALS-linked mutations in *Ubiquilin-2* (*UBQLN2*), and is important for degrading full-length TDP-43 in addition to CTF-35 and CTF-25 [113–120]. Inhibiting this mode of clearance in primary neurons results in a greater accumulation of cytoplasmic TDP-43 aggregates compared to other cell stressors [113, 121, 122]. Recently a gain-of-function mutation in *CYLD* Lysine 63 Deubiquitinase (*CYLD*) was identified to cause ALS and FTL [123]. The authors demonstrated in mouse primary neurons that this mutation increased deubiquitinase activity, decreased autophagy function and caused TDP-43 mislocalization, along with TDP-43 aggregation in the human brain. Autophagy also plays a role in clearing aggregated forms of TDP-43 and is linked to ALS through mutations in autophagy-related proteins *SQSTM1*, *TANK Binding Kinase 1* (*TBK1*) and *Optineurin* (*OPTN*) [87, 113, 124–128]. Of particular importance, the

sequestration of SQSTM1 into TDP-43 aggregates, one of the aforementioned hallmarks of ALS aggregates, leads to the inhibition of proteasome function in addition to autophagy, further promoting the accumulation of toxic, misfolded proteins in cells [129, 130].

The reduced clearance of aggregates can lead to another toxic gain-of-function mechanism: blocking intracellular transport. Aggregates are observed throughout the cytoplasm, often in the soma, but are also observed in the axons and dendrites [131, 132]. Inhibiting axonal transport is a common feature in ALS and particularly relevant as mutations in genes involved in cellular transport, namely *KIF5A* or *DCTN1*, cause ALS [133–139]. This may provide some insight into selective neuron vulnerability in ALS as motor neuron axons are particularly long and susceptible to changes in trafficking dynamics [139]. Additionally, TDP-43 plays an important role in axonal trafficking of mRNA granules, a function lost when it is mutated or aggregated [131, 132, 140–142].

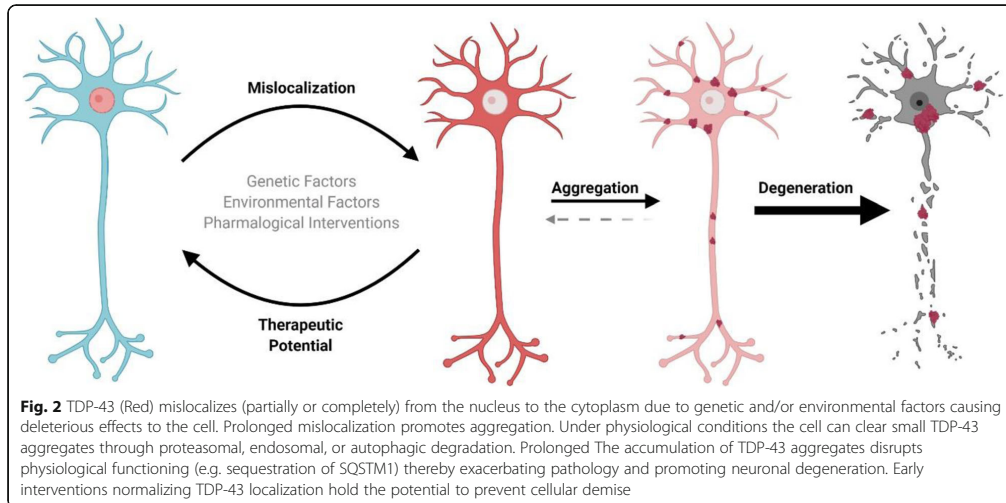
Additional avenues of TDP-43 toxicity

Studies have suggested that not all aggregates are equal in their ability to cause toxicity. Similar to other neurodegenerative diseases, large protein aggregates such as amyloid-like structures may not be as toxic as smaller ones that preceded them such as oligomers [143–147]. However, describing aggregates simply as “large” or “small” is a gross oversimplification as there are thought to be multiple species of aggregates based on the properties of protein misfolding which may mediate altered toxicity at different stages [53, 147–150]. Although TDP-43 aggregation is apparent in various modes of cellular dysfunction, critics argue that TDP-43 aggregates may simply be an artifact of neuronal degeneration observed at the time of post-mortem analyses [14, 151]. In cell and animal models, TDP-43 aggregation is not necessarily essential to cause cellular toxicity [14, 152–157]. This would suggest that TDP-43 aggregates may act as a bystander alongside a cell death pathway or work in parallel with an alternate mechanism to promote toxicity. Furthermore, TDP-43 aggregates are not exclusive to motor neurons, they can also be observed in glia and muscle tissue of ALS patients and are observed to spread in a prion-like manner throughout the brain [8, 9, 15, 57, 158–162]. Yet in ALS, motor neurons selectively degenerate suggesting that the presence of TDP-43 aggregates may not necessarily drive cell-death. Clearly, TDP-43 aggregation is not the only feature at play.

In the presence of ALS-causing mutations, TDP-43 often demonstrates an altered nucleocytoplasmic distribution (increased cytosolic, decreased nuclear) in comparison to its wild-type counterpart [153, 163]. This may suggest that TDP-43 dysfunction can promote cytoplasmic accumulation. However, it remains difficult to differentiate whether

cellular phenotypes may be caused by mislocalization or the mutation itself. Studies have exploited the NLS sequence on TDP-43 through genetic manipulation to shed light on the consequences of mislocalization independent of mutations or aggregate formation as in the cell stress models. In cellular models, expression of TDP-43^{ΔNLS} resulted in depletion of endogenous TDP-43^{WT} from the nucleus and promoted the formation of insoluble inclusions in the cytoplasm [29]. In a transgenic mouse model expressing human TDP-43^{ΔNLS} under a neurofilament heavy chain promoter for brain and spinal cord expression, mice displayed a rapidly progressive motor phenotype, loss of body mass, neuromuscular denervation, and spinal motor neuron loss [155]. These mice also exhibited high levels of phosphorylated TDP-43 aggregates throughout the brain and spinal cord. Interestingly, the authors of this study describe progressive endogenous nuclear TDP-43 depletion followed by aggregate formation in the brain and to a lesser extent the spinal cord. This infers that TDP-43 mislocalization can promote nuclear depletion and is likely upstream of aggregation. Much of the toxicity, however, was attributed to the high level of transgene expression in the animal model which can function to exacerbate the effect of induced TDP-43 mislocalization by inducing cellular stress from TDP-43 overexpression.

The cellular stress model, particularly oxidative stress (e.g. via sodium arsenite treatment), to induce SGs and TDP-43 inclusions is a widely used model to study TDP-43 mislocalization and aggregation [96, 164]. Yet systemic stress makes it difficult to differentiate phenotypes associated with TDP-43 mislocalization and accumulation to general cellular stress responses. To overcome this limitation, a novel model expressed TDP-43 fused with an *Arabidopsis thaliana*-derived Cryptochrome 2 (CRY2) protein to allow for optogenetic instigation of LLPS [108, 109, 111, 165]. In contrast to prolonged sodium arsenite treatment, prolonged LLPS through optogenetic stimulation in wild-type conditions results in TDP-43 inclusions within the nucleus absent of ALS hallmarks including hyperphosphorylation and SQSTM1 sequestration [108]. However, prolonged induction of LLPS on TDP-43^{ΔNLS} or mildly stressed cells inducing a mild mislocalization resulted in cytoplasmic inclusions positive for ALS-like hallmarks. Additionally this model demonstrated in vitro that there is a relatively short time course between initial induction of LLPS to aggregate formation (within hours) and inevitable cell death in less than 6 weeks [108, 111]. This suggests a slippery slope between aggregate formation and neurodegeneration, thereby inferring that therapeutically targeting the aggregation step may be too late to have substantial impact on disease progression (Fig. 2). Increasingly, the field is focusing on mechanisms outside of TDP-43 aggregation to identify early drivers of disease.



The contribution of TDP-43 mislocalization to cellular toxicity in ALS

Increasing evidence suggests that nuclear-to-cytoplasmic mislocalization of TDP-43 induces toxicity through both loss- and gain-of-function mechanisms. Classic roles for TDP-43 pertain to mRNA maturation in the nucleus, specifically acting as a repressor of alternate splicing, cryptic exon splicing, and alternate polyadenylation [25, 166–173]. Loss of these functions through mislocalization or depletion have widespread deleterious effects on the cell [170, 173]. For example, recently it was discovered that loss-of-TDP-43 decreases microtubule outgrowth specifically in motor neurons through premature polyadenylation of the *Stathmin2* (*STMN2*) transcript [167, 174]. TDP-43 is also involved in mRNA transport, a mechanism that is dysregulated within ALS, as well as local translational regulation [131, 175]. Disruption of either of these mechanisms may effectively trap TDP-43 in the cytoplasm, inhibiting its normal functions. This hypothesis is substantiated by transcriptomic evidence showing that diseased neurons and mouse models of ALS demonstrate increases in alternative splicing events, cryptic exon inclusion and alternate polyadenylated sequences [168, 176–178]. Recently, the CTD of TDP-43 was found to mediate its recognition of G-quadruplex structures on RNA, facilitating subcellular transport to neurites for local translation and nucleocytoplasmic trafficking [179, 180]. Interestingly, C9ORF72 hexanucleotide repeat expansion results in G-quadruplex formation, however the relationship between TDP-43 and C9ORF72 in the context of these structures has not yet been explored [181–183]. Additionally, RNA binding

abilities of TDP-43 have been linked to TDP-43 toxicity, though some studies suggest that RNA binding is a protective mechanism [111, 184–189]. An important finding suggests that TDP-43 RNA binding regulates its solubility and lack of RNA promotes aberrant inclusions in the cytoplasm [111, 189]. Mislocalization of TDP-43 may inhibit proper RNA trafficking to the cytoplasm and subsequently promote an environment where TDP-43 is less soluble.

The nuclear functions of TDP-43 are not limited to its RNA binding functions; TDP-43 also binds DNA at TG-rich regions to regulate gene expression and exon skipping [25, 190]. For example, TDP-43 normally binds to the promoter of *Vacuolar Protein Sorting 4B* (*VPS4B*) to repress its transcription [191]. Loss of function due to mislocalization results in a loss of *VPS4B* repression leading to an increased interaction with the ALS-linked protein Charged Multivesicular Body Protein 2B (CHMP2B) thereby disrupting dendritic recycling-endosome trafficking and reducing ALS-linked ERB-B2 Receptor Tyrosine Kinase 4 (ERBB4) surface expression [191–193]. Another nuclear role for TDP-43 is in its response to genomic double stranded breaks (DSBs) which accumulate in ALS patients [163, 194–200]. Mislocalization of TDP-43 through an ALS-causing mutation impair the nuclear localization of DSB-repair proteins and result in the accumulation of DNA damage promoting cell death [163, 194, 201, 202]. Loss of nuclear TDP-43 can also affect chromatin accessibility leading to altered gene expression [203, 204].

Not all consequences of TDP-43 mislocalization are attributed to nuclear loss-of-function as TDP-43 has defined roles in the cytoplasm including stress granule

regulation, mRNA stability, translational regulation, local synaptic RNA regulation, mRNA trafficking, microRNA regulation, and regulation of autophagy (extensively reviewed by Birsa et al. [205]). The exact consequences of increased cytoplasmic TDP-43 on these cellular functions remains largely unknown as most studies focus on protein aggregation resulting in an effective loss-of-TDP-43 function. TDP-43 is cleared through both the ubiquitin-proteasome system and lysosomal degradation pathways (highlighted above) [206–208]. Interestingly, TDP-43 mislocalization through overexpression or pathogenic mutations causes vacuole fragmentation, causing cellular disruption in addition to altering its own clearance [206]. Additionally, mislocalization may prime cells to respond abnormally in certain circumstances. As TDP-43 can readily undergo phase separation, the increase in cytoplasmic density biophysically promotes LLPS to occur (reviewed by Boeynaems et al. [98]). This is apparent in models of cellular stress when TDP-43 is mislocalized as there is a significant increase in the cellular stress response including rapid formation of stress granules and TDP-43 granules [109, 111, 165]. Therefore, mislocalization may sensitize the cell to respond disproportionately to a cellular stress than it may normally be less responsive to. This was recently exemplified in a study where induced pluripotent stem cell (iPSC)-derived motor neurons, but not astrocytes, with mislocalized TDP-43 showed an increase level of cell death when seeded with TDP-43 aggregates from patient tissue [209].

Although we have focused strictly on nuclear and cytoplasmic TDP-43, it is important to highlight a role for TDP-43 at mitochondria. TDP-43 misregulation through genetic manipulation of the NLS, presence of an ALS-causing mutation, or overexpression result in an increased localization to mitochondria [28]. Within the mitochondrion, mutant TDP-43 also preferentially binds mitochondria-resident mRNA, presumably causing Complex 1 disassembly through altered expression of its components [28]. Nevertheless, this finding has been debated, as studies in cell and animal models of ALS suggest that mitochondrial energetics and metabolism are unaltered [210]. The important contribution of mitochondria in ALS however remains a focus due to mutations in the mitochondrial protein SOD1 as a primary genetic cause of fALS [11]. However, it is interesting to consider that fALS caused by mutations in SOD1 rarely present with TDP-43 pathology [15].

Together, these data suggest that both loss- and gain-of-TDP-43 function mediated by nuclear-to-cytoplasmic mislocalization cause systemic cellular dysfunction in ALS. This recognition calls for a better understanding of the native subcellular functions of TDP-43 and the consequences of mislocalization independent of aggregation.

Potential mechanisms driving TDP-43 Mislocalization

It is apparent that TDP-43 mislocalization on its own is toxic and can contribute to many of the cellular characteristics observed in ALS. However, the mechanisms governing TDP-43 localization remain largely elusive. Identifying the mechanisms driving mislocalization will be crucial to identify key mechanisms that are misregulated early in disease and can be therapeutically targeted to prevent TDP-43 pathology all together (Fig. 2). These mechanisms can cover a range of biological aspects intrinsic to TDP-43 function as well as systemic cellular function [202].

As previously described, TDP-43 contains several subcellular-regulatory sequences including an NLS, the controversial NES, and mitochondrial localization sequences M1, M3, and M5. ALS-causing mutations however rarely reside within these motifs (with the exceptions of A90V in the NLS, and mutations between amino acids 294–300 in M5), and TDP-43 mislocalization exists outside of *TARDBP* mutations, suggesting extrinsic factors from TDP-43 govern its subcellular localization [4, 6, 28, 151, 211–222]. Understanding the contribution of these domains to TDP-43 biology remains an important step to understand disease. To this end, targeted mutagenesis of TDP-43 NLS sequence suggests that TDP-43 is actively transported into the nucleus [49]. Furthermore, knock-down of nuclear import machinery (e.g. Importin- β) impair TDP-43 nuclear localization, increasing the cytoplasmic abundance of TDP-43 [223]. Mutagenesis of the NES does not alter TDP-43 localization suggesting the NES is non-functional, however manipulation of export machinery (e.g. Exportin 1) yields conflicting results; thus there may be overlapping mechanisms of TDP-43 export [49–51]. Nevertheless, nuclear pore trafficking is important to some extent for normal TDP-43 localization. Perhaps unsurprisingly, in ALS, nuclear pore trafficking is disrupted, especially in cases of patients bearing mutations in *TARDBP* or *C9ORF72* [223–228]. Aggregates of TDP-43 sequester nuclear pore proteins which would likely exacerbate TDP-43 mislocalization and accumulation into the protein aggregates [224]. In cases of *C9ORF72* repeat expansion, dipeptide repeats (DPRs) generated through repeat-associated non-AUG (RAN) translation of the expanded hexanucleotide (CCCCGG) repeat blocks and disrupts the nuclear pores leading to TDP-43 pathology [229–235]. TDP-43 mislocalization was also shown to exacerbate RAN translation of *C9ORF72* DPRs and could contribute to nuclear pore defects in conjunction or independently from DPRs [235]. This study suggests that *C9ORF72* neurotoxicity may be mediated by TDP-43, and that TDP-43 mislocalization independent of *C9ORF72* DPRs can disrupt nuclear pore function. Thus, nuclear pore complex disruption is an important part of TDP-43 pathogenesis, however, this mechanism may not always precede TDP-43

mislocalization. These studies warrant further investigation into mechanisms that may hinder TDP-43 translocation into the nucleus as potential aggravators of disease.

It is clear that regulation of TDP-43 is crucial for proper function, yet relatively little is known about how TDP-43 is regulated. Post translational modifications play an important role in regulating protein function (Reviewed by Buratti [236]). Along with phosphorylation at S409/410, toxic TDP-43 generally displays an overabundance of phosphate modifications leading to the general consensus that phosphorylation of TDP-43 is toxic [58, 61, 237–245]. However, phosphorylation may play a protective role and promote normal function within the cell [246]. For example, phosphorylation of TDP-43 at T153 and T155 by Mitogen Activated Protein Kinase Kinase (MEK) regulates TDP-43 localization to the nucleolus after heat shock, suggesting a normal maintenance role for phosphorylation in TDP-43 biology [247]. Roles for other post translational modifications such as acetylation, poly-ADP ribosylation (PARylation), oxidation, and ubiquitination have been described suggesting that post translational modifications are likely important for normal TDP-43 function and may have unappreciated roles regulating subcellular localization [112, 236, 248–250].

The role of TDP-43 cleavage into CTF35 and CTF25 is gaining traction as potential contributors of normal and toxic TDP-43 function. CTF35 for example assembles into SGs and plays roles in RNA processing, however, CTF25 does not localize to SGs and remains diffuse throughout the cell [251]. Cytoplasmic localization of CTF35 and CTF25 may be due to the partial and full loss of the bipartite NLS upon cleavage, respectively [59, 63, 252]. Additionally, mutations in TDP-43 and CTF35 also increase mitochondrial localization to the mitochondrial matrix and intermembrane space, respectively [252]. This study highlights that full length TDP-43, not CTF35, may cause oxidative stress, in turn increasing TDP-43 cleavage and promoting mislocalization and aggregation. As these fragments are observed in TDP-43 aggregates, increased in disease, and may induce neuronal toxicity, they remain an interesting mechanism that may provide insight into TDP-43 biology and ALS [64, 77, 251–253]. Several studies have identified alternative spliced isoforms of TDP-43, yet few have been functionally characterized [22, 30, 254]. Recently, a C-terminally truncated alternatively spliced isoform of TDP-43 (“short TDP-43” or sTDP-43) was characterized and found to encode a functional NES within the alternative C-terminus (Fig. 1) resulting in a more cytoplasmic localization compared to TDP-43 [255]. Interestingly, sTDP-43 was upregulated in response to increased neuronal activity, induced mislocalization of endogenous TDP-43, and caused neurotoxicity. Additionally, the sTDP-43 isoform was abundant in TDP-43 aggregates

from ALS patients’ spinal cord and tissue samples. Clearly, focusing exclusively on full length TDP-43 is not encompassing to understand its contribution to ALS. Further understanding the biological roles and consequences of cleaved and alternately spliced forms of TDP-43 will provide novel insight into ALS pathogenesis and aid our interpretations of TDP-43 contributions to disease.

Approaches to study TDP-43 Mislocalization to better understand ALS

As with TDP-43 aggregates, interpreting the extent of TDP-43 mislocalization in patient tissue remains challenging as observations are made post-mortem at the late stage of disease. It may be implied that mislocalization of TDP-43 has occurred where there are cytoplasmic TDP-43 aggregates, however the mechanism(s) of biogenesis of this pathogenic hallmark remain(s) elusive. Future studies should systematically analyze the extent of TDP-43 mislocalization in addition to aggregation in human tissue to gain a better understanding of TDP-43 pathogenesis. Relying on key late-stage hallmarks such as phosphorylation of TDP-43 or SQSTM1 sequestration may limit the understanding of early pathogenesis and may lead to the dismissal of models that do not recapitulate late-stage pathology. Though studies have not systematically analyzed the extent of TDP-43 mislocalization throughout the central nervous system, data suggest that TDP-43 mislocalization correlates with aging in the vulnerable motor neurons of mouse spinal cord tissue [256]. Further understanding the basic biology and general extent of TDP-43 mislocalization throughout the central nervous system will help gain insight into the cell-type specific vulnerabilities to key stages of TDP-43 pathology.

There is a need for developing better models that recapitulate important aspects of ALS, including behavioural, pathological, and molecular phenotypes to further understanding of disease. Although most cases of ALS display TDP-43 proteinopathy, only some transgenic mouse models, and fewer endogenous mouse models, recapitulate TDP-43 pathology and ALS-like phenotypes (ALS mouse models recently reviewed by De Giorgio et al. [257]). An interesting exception is in mice bearing mutations in *Senataxin* (*SETX*), a poorly understood protein thought to act as a DNA/RNA helicase which cause a rare juvenile-onset form of fALS and sALS [258–260]. Both mouse models bearing transgenic and endogenous mutations in *SETX* recapitulate TDP-43 mislocalization, aggregation, and ALS-like phenotypes observed in patients [258]. Within *TARDBP* models, many models that display TDP-43 proteinopathy rely on overexpression approaches. With the advent of genome engineering (e.g. via CRISPR/Cas9) in animals and iPSCs, models have pushed towards studying endogenous TDP-43, moving away from the heavy reliance on

transgenic and toxic overexpression models. Several knock-in mouse models expressing mutant TDP-43 display a range of phenotypes depending on the mutation, but most behavioural phenotypes are quite subtle and occur at a very-late stage [254, 257, 261, 262]. For example, the TDP-43^{Q331K} knock-in mouse model does not display significant motor phenotype whereas the TDP-43^{Q331K} transgenic mouse display some, but not robust, motor deficits [31, 263]. This difference may be due to synergistic effects of the TDP-43 mutation and overexpression in the transgenic model. There are several iPSC models for ALS (recently reviewed by Hawrot et al. [264]) however many of them do not recapitulate key hallmarks of ALS pathology. Neurons derived from TDP-43^{A90V} patient iPSCs display TDP-43 mislocalization (likely due to disruption of the NLS), TDP-43^{M337V} results in slight cytoplasmic granular staining of TDP-43 in iPSC-derived motor neurons, and mislocalization of TDP-43 is observed in TDP-43^{M337V} patient iPSC-derived astrocytes [265–267]. The difficulty in recapitulating TDP-43 pathology may suggest that there are additional mechanisms contributing to ALS in conjunction with TDP-43 mutations, such as changes associated with human aging or chronic stress on the cell. Attempts to exacerbate ALS pathology or behaviour deficits in physiologically relevant models may help to elucidate more complex mechanisms driving disease.

Although protein aggregation has gained the most attention to understand ALS and identify novel therapeutic targets, studying the earlier components of TDP-43 mislocalization may provide an important level of insight into the disease onset and progression. However, studying subcellular localization comes with its own challenges ranging from limitations in technology to convoluted interpretations. TDP-43 mislocalization is primarily studied using cellular fractionation methods or microscopy-based methods. Developing and optimizing more reliable methods to quantitatively analyze TDP-43 subcellular localization will enhance our understanding of critical regulators of TDP-43. For example, identifying nuclear-, mitochondrial-, and cytosol-specific post-translational modifications will allow for the generation of antibodies facilitating more rapid and quantifiable detection of mislocalized TDP-43. These would parallel the antibodies raised against phospho-S409/410 TDP-43 which function as a gold-standard for detection of TDP-43 aggregates through microscopy [61]. The generation of reliable tools will help to resolve potential issues with subjectivity and enhance reproducibility to understand and characterize the consequences of TDP-43 mislocalization. Although much weight is placed on DNA sequencing for modern diagnosis, this technique offers relatively little diagnostic ability in the cases of complex diseases such as ALS. Additionally, DNA sequencing is

generally limited to germline mutations and does not account for potential mosaicism which may arise during an individual's lifetime which could lead to ALS [268]. For example, an ALS patient was reported to express TDP-43 bearing the ALS-causing Q331K mutation specifically in spinal cord neurons, but not in the occipital lobe suggesting this ALS-causing mutation is somatic and thus not likely identified through germline genome sequencing [163]. Biomarkers serve as a potential diagnostic feature in addition to providing insight into disease progression. Current biomarker candidates such as Neurofilament Light Chain are increased in ALS patients and may provide insight into disease progression, but is non-specific to ALS and only provides foresight by about 12 months before symptoms occur [269–273]. As TDP-43 mislocalization is a central feature in ALS and biomarkers based on phenotypes associated with mislocalization may provide the specificity and foreshadowing required for early diagnosis.

Integrative “omic” approaches will be important to identify robust biomarkers capable of diagnosis and providing early insight into disease progression. Determining the direct consequences of TDP-43 mislocalization as they pertain to ALS remains a challenge due to the incomplete understanding of TDP-43 function and general dysfunction associated with disease. Therefore unbiased, systems-based approaches will be important to understand TDP-43 biology surrounding mislocalization. Bulk RNA sequencing has provided great insight into transcriptomic changes in ALS mediated by TDP-43. This technology, however, has limited abilities to detect subtle biologically significant changes that may be cell-type specific. Incorporating single cell RNA sequencing or methods of enriching populations of interest (i.e. flow cytometry, spatial transcriptomics) will help target cell-type specific changes affected by TDP-43 mislocalization in animal and cell models that can translate to human disease [274, 275]. As TDP-43 plays important roles in regulating alternative splicing, alternate polyadenylation, and cryptic exon inclusion, deep RNA sequencing will help identify rare toxic species of RNA that can give insight into ALS progression or lead to biomarker for early disease [276, 277]. Interestingly, mislocalization of FUS was recently identified in iPSCs derived from patients bearing mutations in *Vasolin Containing Protein* (VCP) in addition to spinal cords from sALS patients [278]. FUS mislocalization was suggested to occur due to binding of aberrantly retained introns, namely in the *SEFQ* gene. The authors further suggest FUS mislocalization may be a more common hallmark of ALS than previously recognized however more evidence is required. Proteomic approaches such as immunoprecipitation-mass spectrometry or proximity-labeling mass spectrometry (e.g. APEX Proteomics or BioID) comparing wild-type to

mislocalized TDP-43 will provide insight into locale-specific interactors [224, 279, 280]. Additionally, cross analysis with proteomic data from ALS tissue may provide insight into potential toxic protein-protein interactions as a result of TDP-43 mislocalization serving as early therapeutic targets. New technologies are also focusing on the subcellular localization of RNA such as APEXseq [281–283]. Enhancing this technology with datasets that monitor Protein-RNA binding (e.g. CLIP-seq), could greatly enhance our understanding of which transcripts are bound by TDP-43 in various subcellular compartments by comparing nuclear, cytoplasmic, and mitochondria TDP-43-RNA interactions [284]. Integrating these systems-based approaches will help to uncover novel markers of TDP-43 mislocalization and elucidate pathways leading to cellular demise in ALS.

Conclusions

Increasing evidence suggests that TDP-43 aggregation is not a single driver of pathology in ALS. TDP-43 mislocalization plays significant roles in cellular dysfunction independently and in parallel to aggregation. Increasingly the field has begun to focus on understanding the regulatory mechanisms of TDP-43 mislocalization. To this end, as protein mislocalization is likely more readily reversible than protein aggregation, understanding the mechanisms regulating TDP-43 subcellular localization will be critical for therapy development. Specifically, a better understanding of TDP-43 localization regulators will surely shed light on novel therapeutics that have the potential to be more effective earlier in disease, more generalizable to most ALS cases, and more informative biomarkers for diagnosis and analysis of progression for ALS. Lastly, given that TDP-43 pathology can also coexist with other aggregate-prone proteins, such as C9ORF72 DPRs, Tau, α -Synuclein, and poly-Q expanded Huntingtin, insight into the role of TDP-43 mislocalization in its pathogenic function will serve to better understand pathology and modes of degeneration across a spectrum of neurodegenerative diseases [184, 285–288].

Abbreviations

ALS: Amyotrophic Lateral Sclerosis; C9orf72: Chromosome 9 Open Reading Frame 72; CHMP2B: Charged Multivesicular Body Protein 2B; CNS: Central Nervous System; CRY2: Cryptochrome 2; CTD: C-Terminal Domain; CTF25: [TDP-43] C-Terminal Fragment 25 kDa; CTF35: [TDP-43] C-Terminal Fragment 35 kDa; CYLD: CYLD Lysine 63 Deubiquitinase; DCTN1: Dynactin 1; DSB: Double Stranded Breaks; ERBB4: RB-B2 Receptor Tyrosine Kinase 4 (ERBB4); fALS: Familial ALS; FTL: Frontotemporal Lobar Dementia/ Frontotemporal Dementia; FUS: Fused in Sarcoma; G3BP1: Ras-GTPase Activating Protein-Binding Protein 1; iPSC: Induced Pluripotent Stem Cell; KIF5A: Kinesin Heavy Chain Isoform A; LCD: Low-Complexity Domain; LLPs: Liquid-Liquid Phase Separation; M1-M5: [TDP-43] Mitochondrial Localization Sequence 1-5; MEK: Mitogen Activated Protein Kinase Kinase; NES: Nuclear Export Signal; NLS: Nuclear Localization Signal; OPTN: Optineurin; PARylation: Poly-ADP Ribosylation; PGRN: Progranulin; PrLD: Prion-Like Domain; RAN: Repeat-Associated Non-AUG (translation); RRM: RNA Recognition Motif; sALS: Sporadic ALS/SGS Stress Granule; SOD1: Zn/

Cu Superoxide Dismutase 1; SFPQ: Splicing Factor Proline-, and Glutamine-Rich; SQSTM1: Sequestosome-1; sTDP-43: Short (Alternatively-Spliced) TDP-43; STXN: Senataxin; TARDBP: TAR DNA-Binding Protein 43 kDa (Gene); TBC1D1: TBC1 Domain Family Member 1; TBK1: TANK Binding Kinase-1; TDP-43: TAR DNA-Binding Protein 43 kDa (Protein); TIA1: TIA1 Cytotoxic Granule Associated RNA Binding Protein; UBQLN2: Ubiquilin-2; VCP: Vasolin-Containing Protein; VPS4B: Vacuolar Protein Sorting 4B; WT: Wild Type

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Contribution: I helped perform confocal microscopy, primary neuron cultures, and qPCR experiments. My overall contribution was ~10%.

Constitutive nuclear accumulation of endogenous alpha-synuclein in mice causes motor impairment and cortical dysfunction, independent of protein aggregation

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Abstract

A growing body of evidence suggests that nuclear alpha-synuclein (α Syn) plays a role in the pathogenesis of Parkinson's disease (PD). However, this question has been difficult to address as controlling the localization of α Syn in experimental systems often requires protein overexpression, which affects its aggregation propensity. To overcome this, we engineered *Snca*^{NLS} mice, which localize endogenous α Syn to the nucleus. We characterized these mice on a behavioral, histological and biochemical level to determine whether the increase of nuclear α Syn is sufficient to elicit PD-like phenotypes. *Snca*^{NLS} mice exhibit age-dependent motor deficits and altered gastrointestinal function. We found that these phenotypes were not linked to α Syn aggregation or phosphorylation. Through histological analyses, we observed motor cortex atrophy in the absence of midbrain dopaminergic neurodegeneration. We sampled cortical proteomes of *Snca*^{NLS} mice and controls to determine the molecular underpinnings of these pathologies. Interestingly, we found several dysregulated proteins involved in dopaminergic signaling, including Darpp32, Pde10a and Gng7, which we further confirmed was decreased in cortical samples of the *Snca*^{NLS} mice compared with controls. These results suggest that chronic endogenous nuclear α Syn can elicit toxic phenotypes in mice, independent of its aggregation. This model raises key questions related to the mechanism of α Syn toxicity in PD and provides a new model to study an underappreciated aspect of PD pathogenesis.

Introduction

Alpha-synuclein (α Syn) is a protein notorious for its involvement in Parkinson's disease (PD) pathogenesis. For one, it is a primary constituent of Lewy bodies and Lewy neurites, pathological hallmarks of PD (1). Moreover, copy number variations and missense mutations in the α Syn gene, SNCA, cause genetic forms of PD, further reinforcing its involvement in disease etiology (2–8). α Syn was first described as a presynaptic and nuclear protein (9). However, nuclear α Syn has largely been overshadowed by a focus on its cytoplasmic/synaptic form, likely due to the cytoplasmic localization of Lewy bodies. Despite this, several studies have linked nuclear α Syn to PD on multiple levels: in cell (10–14) and animal (6,15–18) models of PD, as well as in brain tissue from individuals with α Syn pathologies [synucleinopathy; (19–21)]. These studies examined the role of nuclear α Syn by overexpressing it together with mutations or a nuclear localization signal [NLS; (12,15,17,18,22,20)], or upon toxin exposure (16), hinting at a role for nuclear α Syn in disease pathogenesis by its involvement in DNA binding

(23,24) or histone modification (17) to alter transcription, and in DNA repair (25). Although these studies support a link between nuclear α Syn and PD, its specific role in disease—whether deleterious or beneficial—remains clouded due to the reliance on its overexpression or exogenous stressors, making it difficult to parse out the driver of toxicity in the absence of α Syn aggregation.

To directly test the consequence of chronic α Syn mislocalization to the nucleus *in vivo*, without resorting to protein overexpression, we engineered a mouse model to endogenously express α Syn with a C-terminal NLS-Flag tag driving its mislocalization to the nucleus. We extensively characterized these mice at the behavioral, histological and biochemical level to assess whether chronic nuclear localization of α Syn causes age-dependent phenotypes resembling PD or related α Syn proteinopathies.

Results

Effective nuclear targeting of α Syn in *Snca*^{NLS} mice

To study if nuclear α Syn is sufficient to elicit age-related behavioral and pathological phenotypes, we generated a

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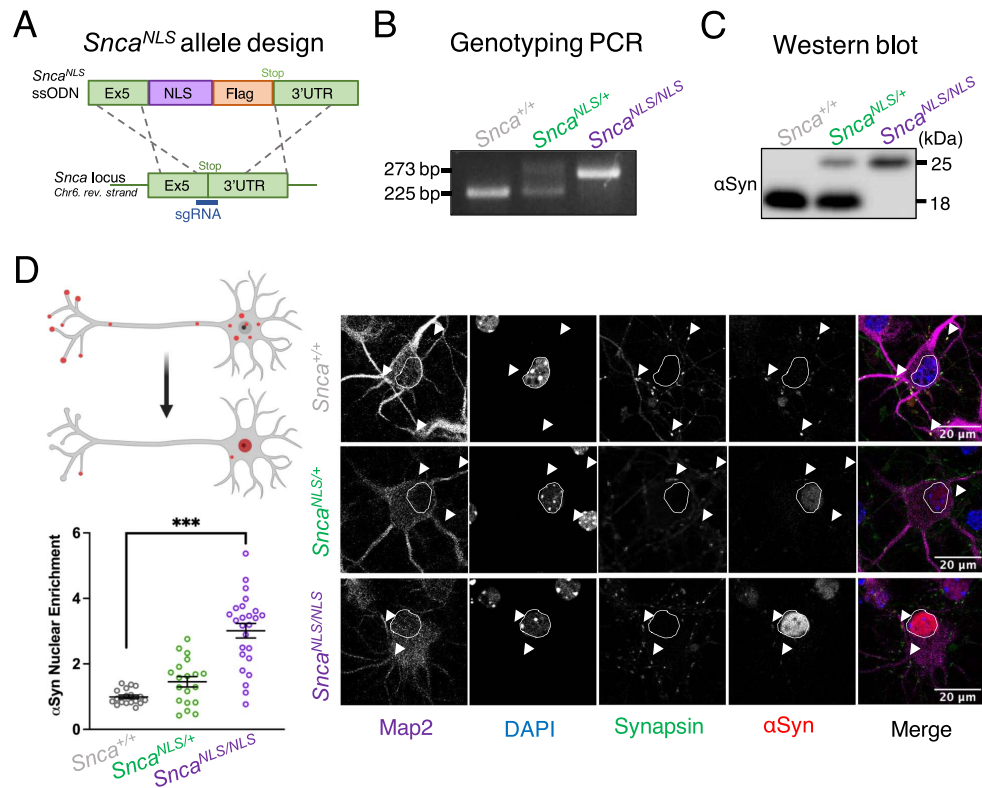


Figure 1. *Sncα*^{NLS} mice effectively target αSyn to the nucleus in vitro and in vivo. (A) *Sncα*^{NLS-Flag} knock-in scheme with C-terminal NLS-Flag tag. Visualization of the knock-in via (B) PCR and (C) western blot. (D) Illustration of nuclear localization of αSyn (upper left) with quantification (lower left) of nuclear αSyn from primary cortical neurons in wildtype (top right panels), *Sncα*^{NLS/+} (middle right panels) and *Sncα*^{NLS/NLS} (bottom right panels; n = 3). Protein quantification (lower left) depicts nested relative per-cell intensity of nuclear αSyn from three independent experiments. White arrows denote presynaptic αSyn and nuclei are circled in white. Nested one-way ANOVA with Bonferroni multiple comparison: ***denotes P < 0.001.

mouse line that targets endogenous αSyn to the nucleus via the knock-in of an NLS-Flag tag on αSyn (*Sncα*^{NLS}). The NLS-Flag construct was targeted to the 3' end of the *Sncα* coding sequence with the modified *Sncα*-NLS-Flag gene predicted to transcribe a fusion protein of wildtype αSyn with a C-terminal NLS-Flag tag (Fig. 1A). After generating and backcrossing mice (see Materials and Methods and Supplementary Material, Fig. S1), we confirmed the knock-in via sequencing (Supplementary Material, Fig. S1B) and were able to distinguish between the genotypes via a PCR band shift (Fig. 1B) and a larger protein size via western blot (Fig. 1C). Mice were born at expected Mendelian ratios (Supplementary Material, Fig. S1C), confirming that insertion of this tag did not pose major developmental deficits.

To examine the efficiency of the NLS-Flag tag, we cultured primary cortical neurons for 7 days in vitro and quantified the level of nuclear αSyn through immunofluorescent microscopy. We observed a 3-fold increase in nuclear αSyn in *Sncα*^{NLS/NLS} and a 1.5-fold increase in *Sncα*^{NLS/+} compared with *Sncα*^{+/+} (wildtype) cells (Fig. 1D). This trend was consistent in stained adult

mouse brain tissue at both 2- and 18-months where we found a 2–5-fold increase in nuclear αSyn in the cortex (Fig. 2A), dentate gyrus (Fig. 2B) and substantia nigra (Fig. 2C). Importantly, this roughly corresponds to the 2.5–3-fold increase of nuclear αSyn, which we and others have previously observed in post-mortem brain tissue from individuals with PD or other animal models of synucleinopathy, suggesting the model displays a disease-relevant increase of nuclear αSyn (6,15–19).

Increased nuclear αSyn leads to an age-dependent motor decline

To test whether chronic nuclear accumulation of αSyn is sufficient to elicit PD-like phenotypes over time, we subjected *Sncα*^{NLS/NLS} mice and littermates to a battery of behavior tests at 3-, 9- and 18-months of age. We found that the *Sncα*^{NLS/+} and *Sncα*^{NLS/NLS} mice performed similarly to wildtype at 3-months of age (Fig. 3A). By 9-months, however, the *Sncα*^{NLS/NLS} mice displayed a significant motor deficit in rotarod (Fig. 3B) as well as a delayed time to contact their forepaws in the adhesive

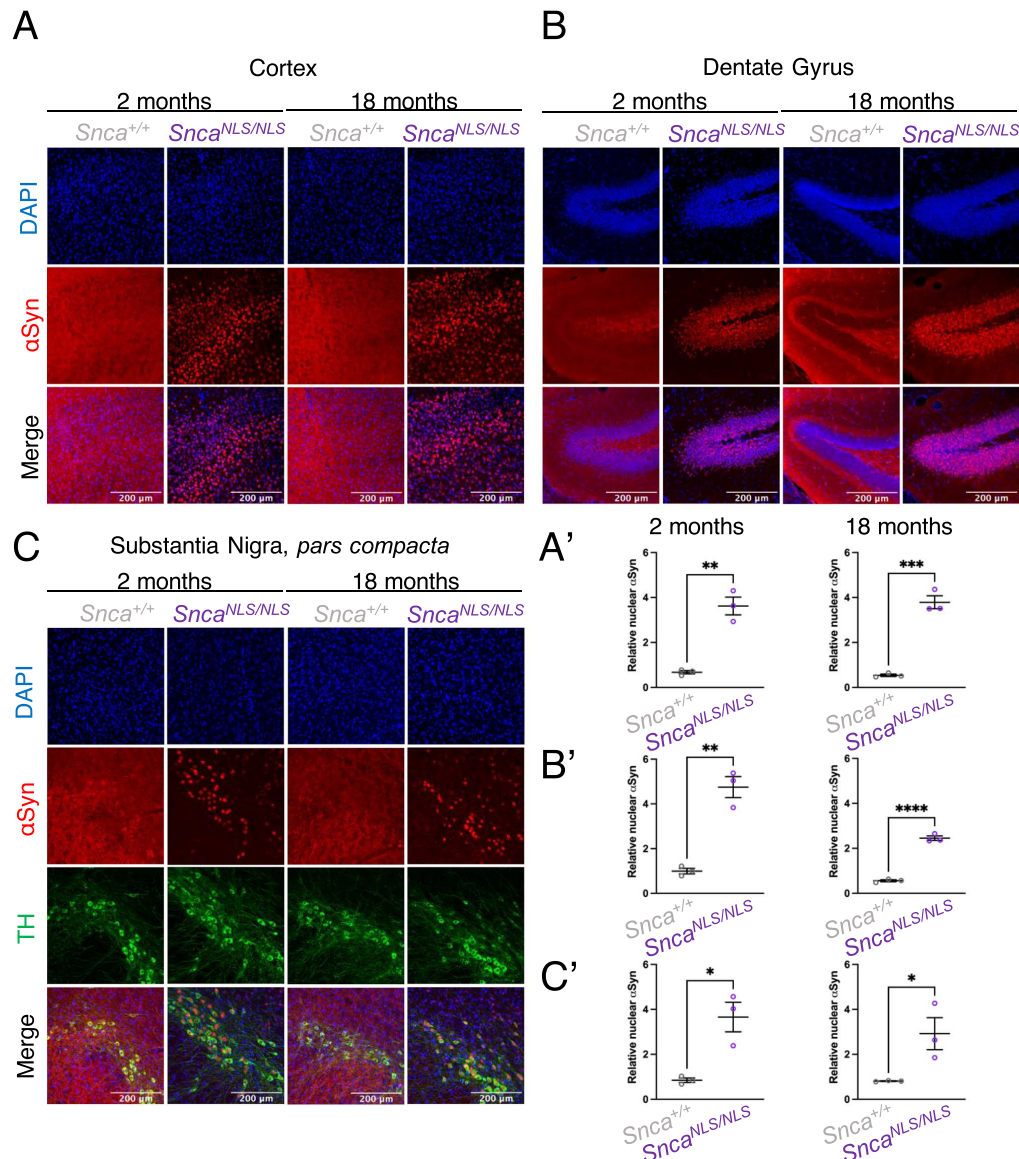


Figure 2. *Snca*^{NLS} mice exhibit a consistent 2–5-fold increase in nuclear α Syn with age. Immunofluorescent images of 2- (left) and 18-month (right) mouse (A) cortex, (B) dentate gyrus and (C) substantia nigra, *pars compacta*. Nuclear enrichment of α Syn was quantified for (A'–C') all brain regions at both 2- (left) and 18-months (right). Unpaired, two-tailed t-test: *, **, ***, **** denote $P < 0.05$, < 0.01 , < 0.001 and < 0.0001 , respectively.

test (Fig. 3C). Interestingly, *Snca*^{NLS/+} mice also exhibited a significant deficit on the rotarod test—suggestive of a dominant phenotype. This rotarod deficit was not observed when testing *Snca*^{-/-} mice at 9 months (Supplementary Material, Fig. S2), suggesting it is due to the accumulation of nuclear α Syn and not a loss-of-function of pre-synaptic α Syn. Surprisingly, after aging the *Snca*^{NLS} mice to 18 months, we observed milder

motor phenotypes relative to wildtype controls; likely due to the 18-month wildtype mice showing increased difficulty at performing these tasks when comparing their motor ability with that at 3- and 9-months of age (Supplementary Material, Fig. S3).

With increasing awareness around non-motor symptoms in PD, we also measured cognition, anxiety and overall wellness in the *Snca*^{NLS} line. We found that

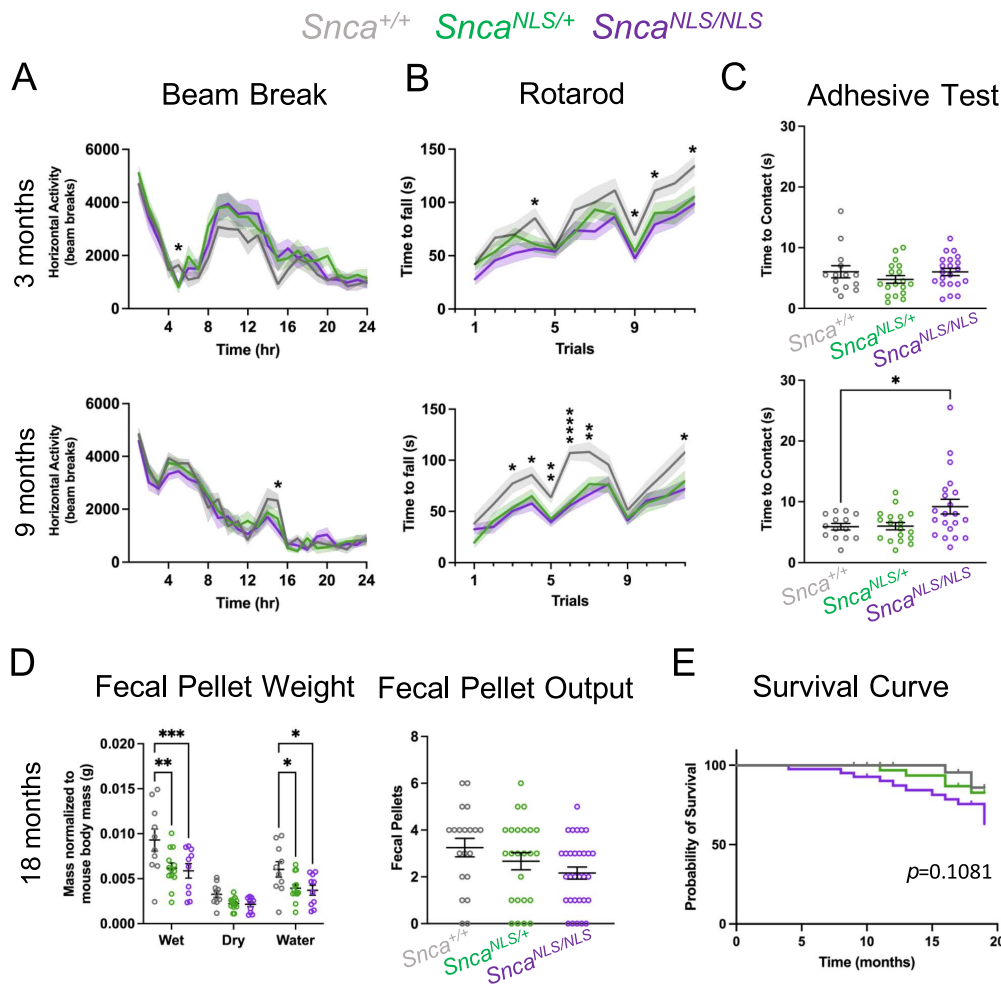


Figure 3. *Snca*^{NLS} mice exhibit significant motor and gastrointestinal dysfunction. Analysis of the 3- (top) and 9-month (bottom) mice for (A) Beam Break, (B) Rotarod and (C) Adhesive Test measuring time to contact their forepaws, (D) Fecal Pellet Weight measuring fecal weight and water content over 1 h (left), and Fecal Pellet Output measuring fecal pellets produced in 10 min (right), ($n=10-21$). (E) Survival curve from all mice in the behavior colony ($n=42-58$). One-way (C, D right), two-way ANOVA (A, D left), or mixed-effects analysis (B) with Bonferroni multiple comparison or Log-rank (Mantel Cox) test (F): ns, *, **, *** and **** denote $P > 0.05$, < 0.05 , < 0.01 , < 0.001 and < 0.0001 , respectively.

Snca^{NLS/NLS} and *Snca*^{NLS/+} mice performed similarly to their wildtype littermates in non-motor behavior tests at all timepoints (Supplementary Material, Figs. S4-S6). In addition to motor decline, people living with PD often experience gastrointestinal difficulties such as constipation (26,27). To measure constipation in our mice, we examined fecal excretions in the span of an hour. We found that 18-month-old *Snca*^{NLS/NLS} mice excretions contained significantly less water than their wildtype counterparts (Fig. 3D). Lastly, we observed a trend for early lethality in the *Snca*^{NLS/NLS} mice, whereby 37% of *Snca*^{NLS/NLS} mice died by 20 months of age, compared with 14% of wildtype littermates ($P=0.1081$,

Fig. 3E). Taken together, the *Snca*^{NLS/NLS} mice display age-dependent motor and gastrointestinal dysfunction reminiscent of PD.

***Snca*^{NLS/NLS} mice exhibit cortical atrophy, independent of α Syn aggregation or dopaminergic neurodegeneration**

Many studies suggest that α Syn toxicity is intrinsically tied to its aggregation, as the two are often associated in humans with PD and in animal models of the disease (1,28,29). However, models of α Syn toxicity often rely on the introduction of synthetically derived misfolded α Syn fibrils (30,31) or overexpression of α Syn (15,32), thereby

potentiating its aggregation *in vivo*. Given that the *Snca*^{NLS} mice display age-dependent behavioral phenotypes, yet do not rely on α Syn overexpression, we asked whether accumulation of endogenous α Syn in the nucleus leads to its aggregation and thusly contributes to its toxicity. We examined both the solubility of α Syn as well as its pathologically linked phosphorylation at serine residue 129 (pS129) by biochemical fractionation of brain samples of *Snca*^{NLS/NLS} mice compared with littermates. We used the *mThy1-SNCA* ('line 61') transgenic and *Snca* knockout (*Snca*^{-/-}) mouse lines as positive (32) and negative (33) controls, respectively. To our surprise, we found that the accumulation of nuclear α Syn does not lead to aggregation (Fig. 4A), nor does it become phosphorylated at S129 (Fig. 4A, B), and in fact total α Syn levels are reduced in these mice (Fig. 4A–C). These findings were further supported by histology, which showed no marked increase in pS129 in aged *Snca*^{NLS/NLS} mice compared with their respective littermates (Fig. 4D). This suggests that nuclear accumulation of α Syn confers neuronal dysfunction independent of aggregation.

A hallmark of PD is nigrostriatal degeneration. Because of the relatively high expression of α Syn in dopaminergic neurons [Fig. 2C; (34)], we hypothesized nuclear α Syn could be acutely toxic to dopaminergic neurons, causing their death and ultimately leading to the observed behavioral deficits in *Snca*^{NLS} mice. To our surprise, we found that young and aged *Snca*^{NLS/NLS} mice had intact nigrostriatal tracts, when evaluated by striatal tyrosine hydroxylase (TH) fiber density and stereological estimation of dopaminergic cell number in the SNc at 3- and 18-months of age (Fig. 5A and B, Supplementary Material, Fig. S4E). Moreover, HPLC (high-performance liquid chromatography) analysis of mouse striata revealed that 18-month-old mice across genotypes exhibit similar levels of dopamine and its metabolites (DOPAC, HVA and 5-HIAA; Fig. 5C).

Since *Snca*^{NLS/NLS} mice exhibit motor defects without nigrostriatal degeneration nor α Syn aggregation, we took a step back to ask whether nuclear α Syn may impact other areas of the brain, thus contributing to PD-like phenotypes. Cortical involvement has long been linked to several synucleinopathies including PD, dementia with Lewy bodies (DLB) and PD with dementia [PDD; (29,35–37)]. We therefore explored higher order cortical areas to determine whether *Snca*^{NLS/NLS} mice exhibit neurodegenerative features outside of the SNc. We conducted gross anatomical studies using hematoxylin and eosin (H&E) and toluidine blue staining and found significant anterior cortical thinning in the motor cortex (Fig. 6A) and a marked increase in pyknotic cells (Fig. 6B) throughout the cortex of 18-month-old *Snca*^{NLS/NLS} mice. We further stained cortical sections with NeuN [Neuronal nuclei antigen, a.k.a. RNA Binding Fox-1 Homolog 3 (Rbfox3)] and found a significant decrease in neurons in layers V/VI of 18-month-old *Snca*^{NLS/NLS} mice compared with their littermates (Fig. 6C). Thus, nuclear α Syn causes

cortical dysfunction in the absence of its aggregation or nigrostriatal degeneration.

Unbiased proteomic analysis uncovers reduced Darpp-32, Pde10a and Gng7 levels in *Snca*^{NLS/NLS} mice

We followed an unbiased strategy to uncover the molecular mechanisms underlying the behavioral and histological phenotypes of the *Snca*^{NLS/NLS} mice via quantitative proteomic analysis on cortices from 9-month-old mice. At this age, *Snca*^{NLS/NLS} mice exhibit behavioral abnormalities (Fig. 3), allowing identification of early molecular changes that drive the late-stage cortical atrophy exhibited in these mice and in PD. To quantify proteomic differences in wildtype and *Snca*^{NLS/NLS} mice, we performed pooled TMT10plex labeling for 5 wildtype and 5 *Snca*^{NLS/NLS} mouse cortices followed by mass spectrometry to identify differential proteomic changes (Fig. 7A). This approach yielded a list of nearly 1800 proteins, of which 114 had a Log₂ fold-change of ± 1 relative to wildtype (Fig. 7B). Of these 114 hits, 66 were downregulated and 48 were upregulated (Supplementary Material, Table S2). Gene ontology term analysis revealed a significant enrichment for biological processes that are disturbed in PD, including regulation of GPCR signaling (Fig. 7C). To increase the stringency of our list, we filtered these hits using a statistical cut-off (Mann-Whitney P-value < 0.05). From this, we identified 10 high-confidence hits (Fig. 7D). Interestingly, among these 10 hits we noticed a few proteins of particular importance in DA signaling and have been associated with PD, such as *Cacna1e*, *Darpp-32* [dopamine- and cAMP-regulated neuronal phosphoprotein a.k.a. protein phosphatase 1 regulator inhibitor subunit 1b (Ppp1r1b)], *Fgf1*, *Gng7* (G Protein Subunit Gamma 7), *Pde10a* (Phosphodiesterase 10A) and *Serpina1a* (38–45). We confirmed overall trends in reduction of *Darpp-32*, *Pde10a* and *Gng7* in the 9-month-old *Snca*^{NLS/NLS} mice using western blot (Fig. 7E), thereby validating our proteomics approaches. Collectively, these data suggest that disrupted dopaminergic signaling pathways may be an early event of nuclear α Syn toxicity.

Discussion

The mechanisms underlying α Syn toxicity have been difficult to pin down. We and others have previously shown that nuclear α Syn is increased in the PD post-mortem brain and in animal models harboring SNCA mutations (6,13,20,19,46). Nonetheless, previous studies examining the role of nuclear α Syn in PD pathogenesis have yielded conflicting results, ranging from neurodegenerative (13,15,17,46) to neuroprotective (20,25) phenotypes. This may be in part due to the degree of overexpression of α Syn or the choice of read-out in these models. Our study sought to overcome this by answering if the nuclear accumulation of native α Syn is sufficient

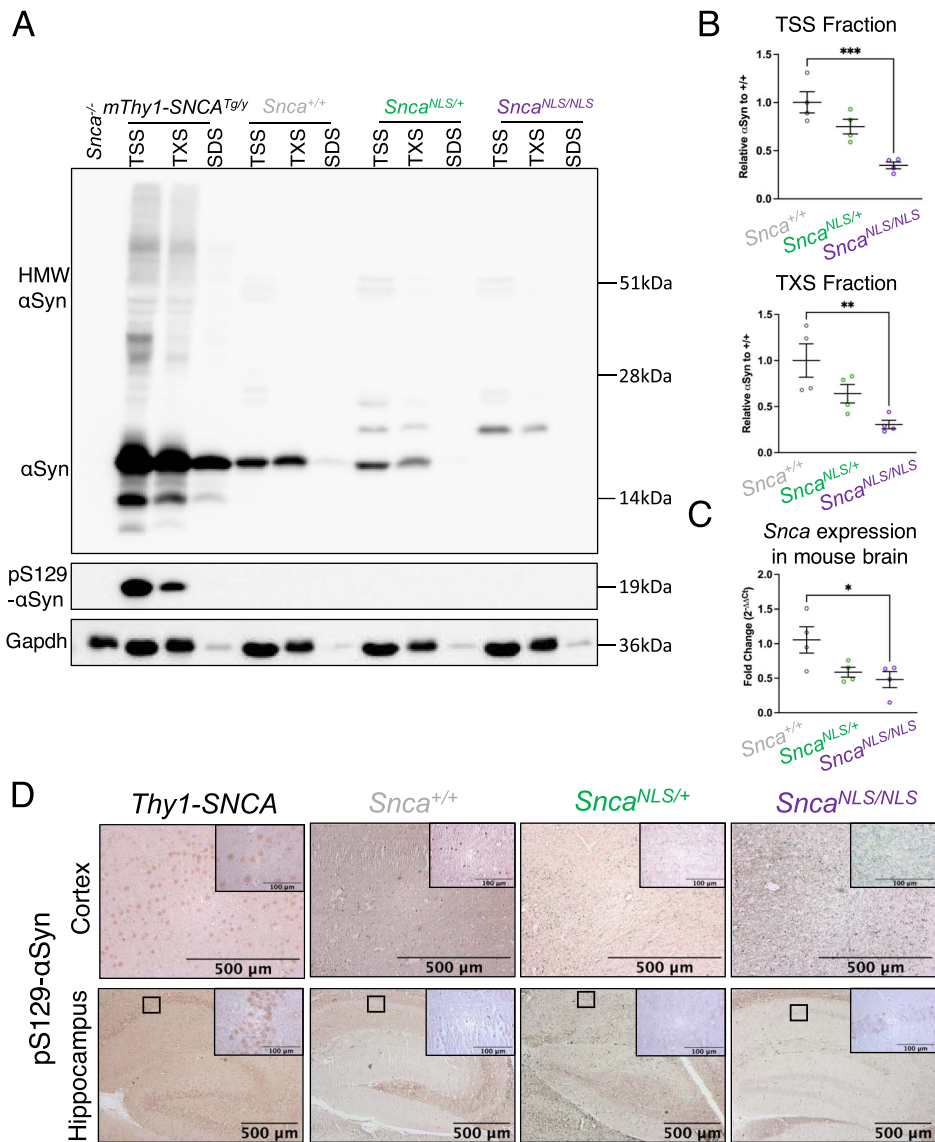


Figure 4. *Snca*^{NLS/NLS} mice do not display significant changes in aggregated or phosphorylated α Syn. Serial extraction of cortical mouse brain tissue with western blot probed for (A) α Syn (upper blot) and pS129- α Syn (middle blot) and Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh; bottom blot) comparing the level of α Syn in the (B) TSS (upper) and TXS (lower) fraction ($n=4$). (C) qPCR of *Snca* mRNA from 9-month-old mouse cortex ($n=4$). (D) pS129- α Syn staining of the motor cortex (upper) and hippocampus (lower). One-way ANOVA with Bonferroni multiple comparison: *, ** and *** denotes $P < 0.05$, < 0.01 and < 0.001 , respectively.

to cause PD-like phenotypes in mice. We engineered a mouse model with an NLS-Flag knockin on *Snca* to characterize the effects of chronically increased nuclear α Syn. We found that *Snca*^{NLS/NLS} mice reveal PD-like phenotypes including age-dependent motor decline, as well as cortical dysfunction. The cortical atrophy we observed

draws parallels to the cell loss seen in synucleinopathies with cortical involvement like DLB and PDD (29,35–37). Moreover, the anatomical location of this cell loss dovetails with the motor deficits seen in these mice and may shed light onto how nuclear α Syn in PD may be linked to cortical dysfunction and disease manifestation. When

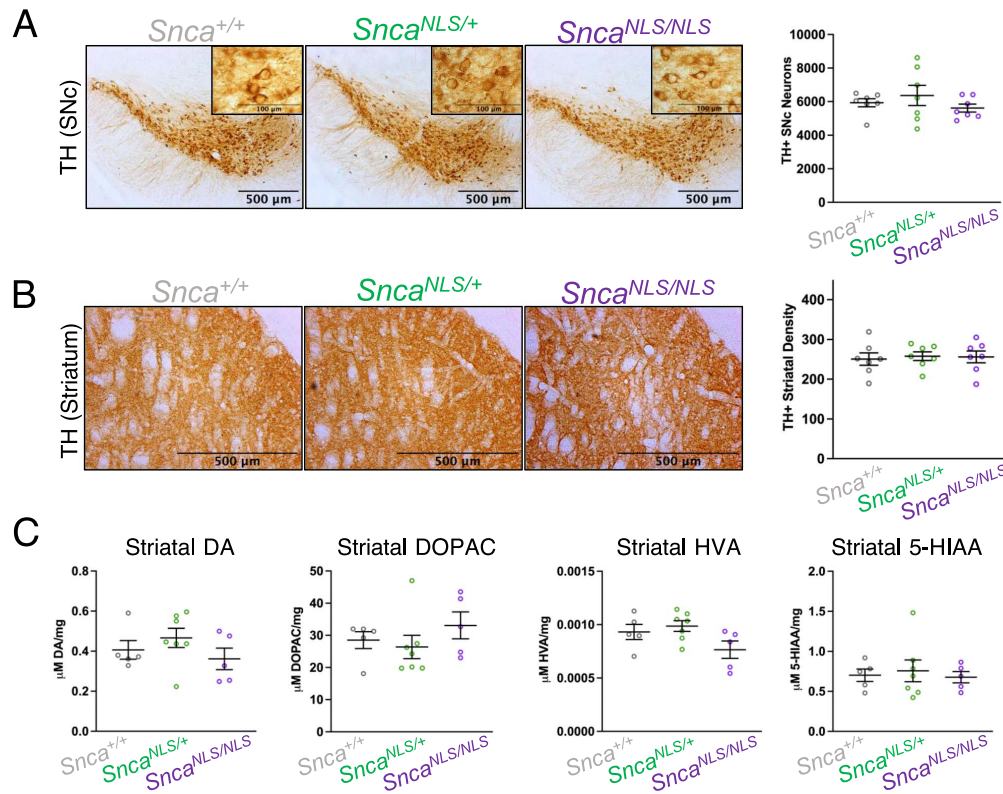


Figure 5. 18-month-old *Snca*^{NLS/NLS} mice exhibit intact nigrostriatal tracts. Tyrosine hydroxylase staining of the (A) Substantia nigra pars compacta and (B) striatum of 18-month mice (*n* = 7). (C) HPLC of 18-month striatal tissue measuring Dopamine (left), DOPAC (middle left), HVA (middle right) and 5-HIAA (right) (*n* = 5–7). One-way ANOVA with Bonferroni multiple comparison: ns denotes *P* > 0.05.

examining the proteomic profile of the *Snca*^{NLS/NLS} mice, we found a few high-confidence hits that have been previously associated with PD. Among these, we identified Pde10a, Darpp-32 and Gng7. Although we found no significant decrease in striatal dopamine, it is interesting to note that these proteins are all involved in dopamine signaling, hinting at a potential mechanism by which nuclear α Syn causes dysfunction via perturbing intracellular signaling (38,42,47–55). Importantly, *Gng7* knockout mice exhibit significant age-dependent motor deficits, particularly in the rotarod test which was the motor assay with which the *Snca*^{NLS} mice exhibited the most difficulty (49). In addition, one study looking at patients with schizophrenia found reduced Pde10a and medial prefrontal cortex thinning, another aspect observed in the *Snca*^{NLS} mice (55). Together, these changes in protein levels hint at a possible mechanism whereby nuclear α Syn elicits dopaminergic signaling defects, in the absence of overt dopaminergic neurodegeneration.

Although characterizing *Snca*^{NLS/NLS} mice, we consistently noted how this mouse line diverges from *Snca*^{-/-} mouse phenotypes (Supplementary Material,

Fig. S2), cementing that *Snca*^{NLS/NLS} mouse phenotypes are likely gain-of-function. To wit, *Snca*^{-/-} mice exhibit mild synaptic deficits in the absence of gross motor or non-motor deficits (33,56), likely due to compensation by β -synuclein and, to a lesser extent, γ -synuclein (57–59). Indeed, the motor phenotypes appear to be dependent on the local dose of nuclear α Syn as even the *Snca*^{NLS/+} mice exhibit some motor behavior deficits—albeit to a lesser extent than their *Snca*^{NLS/NLS} littermates, suggesting that they are due to a gain-of-function of nuclear α Syn and not a loss-of-function of synaptic α Syn. Nevertheless, we cannot exclude a model in which partial loss of synaptic α Syn combined with increased nuclear α Syn may drive the age-dependent behavioural and pathological phenotypes seen in *Snca*^{NLS} mice. Strikingly, behavioral and histological phenotypes in *Snca*^{NLS} mice occur independently from α Syn aggregation and pathogenic phosphorylation. Indeed, overall levels of α Syn are reduced in *Snca*^{NLS} mice—potentially by some regulatory mechanism or feedback loop—therefore suggesting that these α Syn-mediated phenotypes occur despite its aggregation or phosphorylation. This suggests

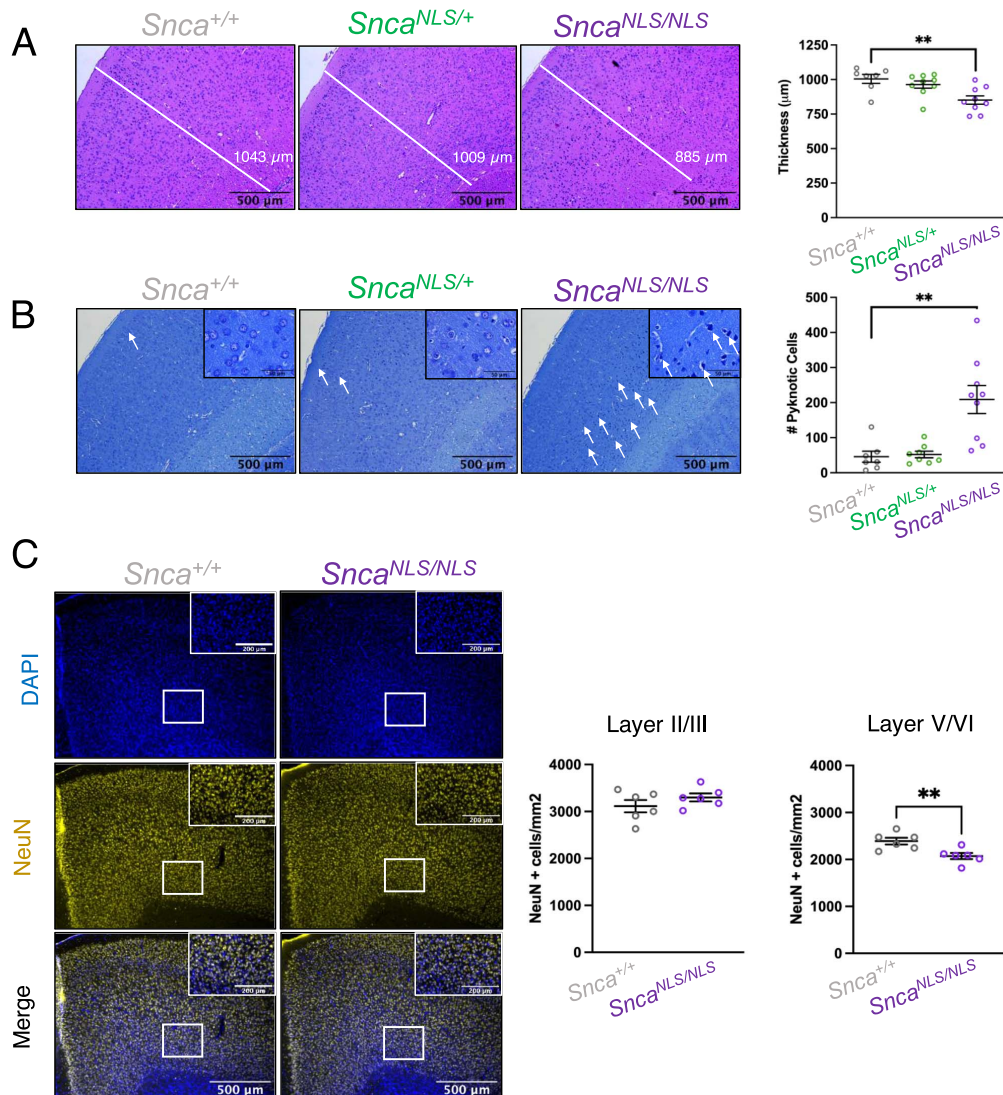


Figure 6. Aged *Snca*^{NLS/NLS} mice exhibit cortical atrophy. (A) H&E staining with quantification of the motor cortex thickness ($n = 7-9$). (B) Toluidine blue staining with quantification of pyknotic cells from the motor cortex ($n = 7-9$). White arrows denote select pyknotic cells. (C) NeuN staining of wildtype (left) and *Snca*^{NLS/NLS} (right) measuring NeuN⁺ cells in motor cortex layers II/III and V/VI. One-way ANOVA with Bonferroni multiple comparison (A, B) or unpaired, two-tailed t-test (C): ns, ** denote $P > 0.05$ and < 0.01 , respectively.

a heretofore underappreciated role of soluble, nuclear α Syn in the pathogenesis of PD.

The cellular mechanisms that drive the nuclear accumulation of α Syn and its subsequent sequelae in PD remain elusive. Whether active or passive mechanisms bring α Syn to the nucleus is unknown. Native α Syn does not possess an NLS, therefore, it may be driven into the nucleus by passive mechanisms [17]

can traverse the nuclear pore complex due to its small size; [60]] and could be retained by interaction with nuclear components (e.g. histones or DNA) [16,17,20,24,25,61] or via uncharacterized modifications. Alternatively, active mechanisms such as its interaction with TRIM28 [19] or RAN [14] may be key in regulating its nuclear import. Moreover, α Syn likely has an important native role in the nucleus, particularly during

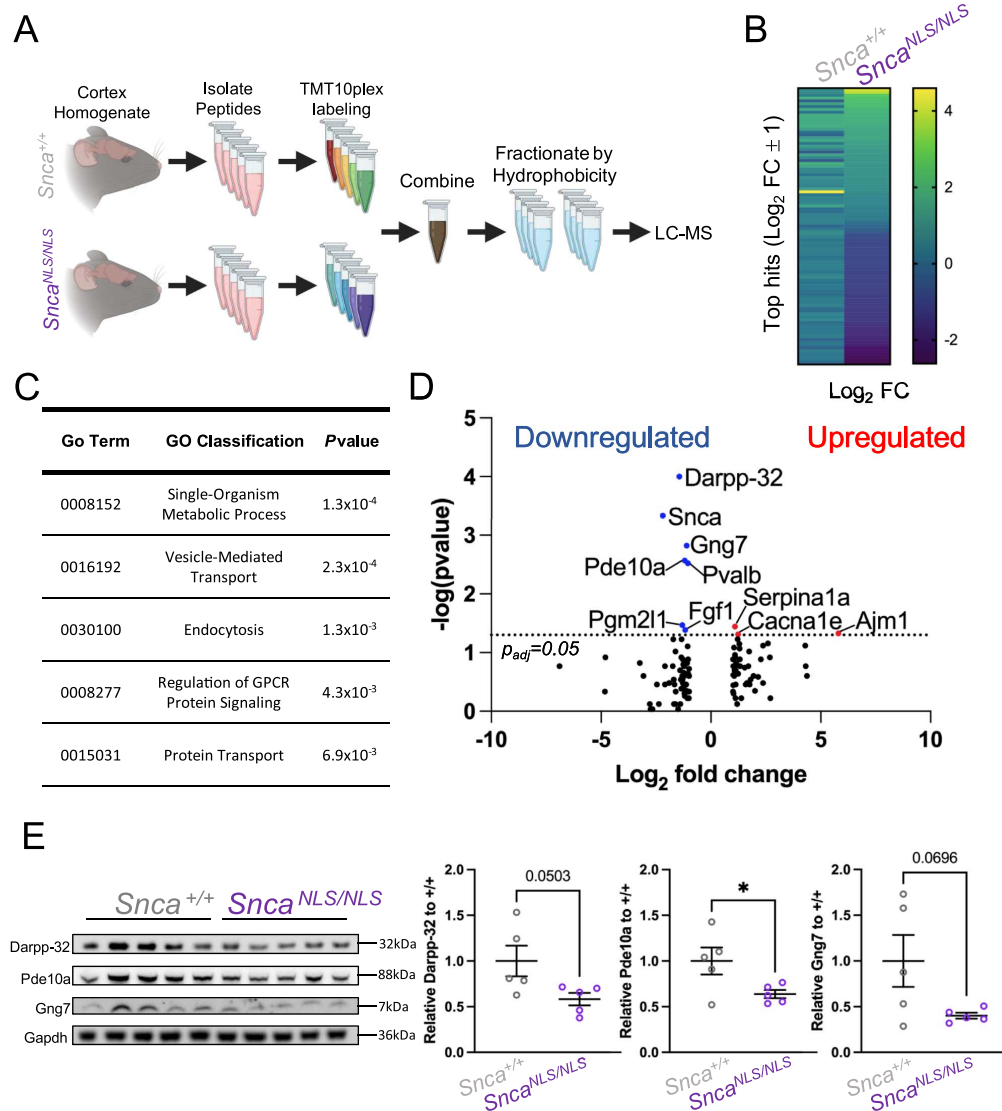


Figure 7. Mass spectrometry reveals proteomic alterations in the *Snca*^{NLS/NLS} mice. (A) Mass spectrometry scheme for quantitative comparison of proteome between 9-month wildtype and *Snca*^{NLS/NLS} cortices ($n=5$). (B) Heat map of all proteins identified through mass spectrometry with values within ± 1 Log₂ fold-change. (C) Table of enriched gene ontology pathways among the ± 1 Log₂ fold-change hits. (D) Volcano plot of mass spectrometry results with ± 1 Log₂ fold-change highlighting significantly upregulated proteins in red and downregulated in blue. (E) Western blot of cortical mouse brain tissue from 9-month mice probing for Darpp-32 (upper), Pde10a (upper middle), Gng7 (lower middle) and Gapdh (bottom) with quantification (right). Unpaired, two-tailed t-test: * denotes $P < 0.05$.

mouse embryonic development, where nuclear α Syn constitutes up to 40% of its total cellular distribution, compared with 3–15% of total cellular distribution in adult mice (20). There, α Syn is suggested to bind both DNA and histones to modulate gene expression (16,17,24,25,61–64). In wildtype mice, nuclear α Syn was shown to be neuroprotective by binding to DNA

and colocalizing with DNA damage response elements to protect against DNA damage (25). Whether the increase in nuclear α Syn observed in PD—modeled in the *Snca*^{NLS} mice—causes a gain of this normal developmental function or a neomorphic function will be important to establish, to facilitate future therapeutic development.

Materials and Methods

Mouse design and engineering

Mouse engineering

To generate the *Snca*^{NLS} mice, Cas9 protein was complexed to a sgRNA targeting the 3' locus of *Snca* (sgRNA target sequence: 5'-TTGGTAGCCTTCCTAATATC-3'); and, together with a single-stranded oligodeoxynucleotide (ssODN) repair template (sequence: 5'-CACTGTGAAGC-AGACAGTTGATATCTGTCACTTCACTGACAAGGCATGCT-GTTATTATTTCTTTTCTGATATTAGGAAGGCTACCAAG-ACTATGAGCCTGAAGCCGACTACAAGGACGACGACGACC-AAGTAAGAATGTCATTGCACCCAATCTCCTAAGATCTGC-CGGCTGCTCTCCATGGCGTACAAGTGCTCAGT-3'; IDT Ultramer), were injected and implanted into pseudo-pregnant FVB female mice (65). Three founder mice were generated and backcrossed onto a ~99.75% pure C57Bl6/J background (Taconic 1450 SNP analysis, sequencing and subsequent backcrossing; see [Supplementary Material, Fig. S1A and B](#)), before being expanded onto a mixed C57Bl6/J; C57Bl6/NCrI background. One line was selected for subsequent extensive characterization and is being made available through the Jackson Laboratory (RRID:IMSR_JAX:036763).

Genotyping

A small (~1 mm) tail sample is digested prior to PCR amplification using primers outside of the sequence covered by the ssODN used for the initial mouse line. Forward: 5'-TTTTATCTGATTGAAATGATGAGC-3'; Reverse: 5'-ATGACTGGGCACATTGGAA-3'. PCR protocol: 95°C for 2 min, (95°C for 30 s, 56°C for 30 s, 72°C for 30 s) repeated for 35 cycles, 72°C for 5 min. Mutant allele: 273 bp; Wildtype allele: 225 bp.

Mouse husbandry

All mice were housed with up to 5 mice per cage on a 12 h light-dark cycle. Mice were fed *ad libitum* and all husbandry was performed by the uOttawa Animal Care and Veterinary Services staff. All animal work was done under the approved breeding (CMMb-3009 and CMMb-3654) and behavior (CMMe-3091) protocols approved under the uOttawa Animal Care Committee. All mice were handled daily for 1 week prior to all behavior testing and both male and female mice were used in all experiments.

Behavior

Open field

Lighting in the behavior room was set to 100 lux and mice were habituated for 60 min prior to testing. Mice were placed into the open field box (45 cm³) for 10 min with their movement recorded/analyzed with Ethovision software (Noldus Information Technology). [dx.doi.org/10.17504/protocols.io.b5qzq5x6](https://doi.org/10.17504/protocols.io.b5qzq5x6).

Fecal pellet output

Upon completion of the Open Field test, the number of fecal pellets excreted during the 10 min trial were quantified.

Beam break

Single mice were placed into a clean cage with access to food and water *ad libitum* for 24 h at the standard 12 h light-dark cycle with their movement recorded/analyzed via Fusion software.

Nesting: Directly following Beam Break testing, one square of nestlet (5 cm² cotton pad) was placed in each Beam Break cage for 17–19 h. Following this, the nestlets were scored on a scale of 1–5 as described in Deacon 2006 (66), with 1 and 5 representing minimal and maximal nest quality, respectively. [dx.doi.org/10.17504/protocols.io.b5q2q5ye](https://doi.org/10.17504/protocols.io.b5q2q5ye).

Y maze forced alternation

Mice were provided with extra-maze (irregular black cue with squared edges on right wall, black triangle on left wall) and intra-maze (Arm 1 has solid black rectangle, Arm 2 has horizontal bars and Arm 3 has diagonal stripes) cues. The room was set to 60 lux and mice were habituated for 60 min prior to testing. During the first 5 min trial, Arms 2 and 3 were alternately blocked. Following the 30 min inter-trial interval, the mice were placed back into the Y maze apparatus for 5 min without any blocked arms and their movements were recorded/analyzed with EthoVision software. [dx.doi.org/10.17504/protocols.io.b5smq6c6](https://doi.org/10.17504/protocols.io.b5smq6c6).

Adhesive test

After a 60 min habituation, the home cage of the mice was lightly wiped to remove all bedding material. The mice were individually placed back into the emptied home cage for a 1 min habituation. Next, a 1 cm² of medical adhesive was placed on each forepaw and the mice were placed back into the wiped home cage where the time to remove adhesive was measured up to a 2 min maximum. [dx.doi.org/10.17504/protocols.io.b5snq6de](https://doi.org/10.17504/protocols.io.b5snq6de).

Pole test

Following a 60 min habituation, mice were placed on a textured metal pole (8 mm diameter, 55 cm tall) ~3 cm from the top facing upwards. The mice were given up to 1 min to turn around (facing downwards) and up to 1 min to descend the pole. [dx.doi.org/10.17504/protocols.io.b5sqq6dw](https://doi.org/10.17504/protocols.io.b5sqq6dw).

Rotarod

Following a 60 min habituation, mice were placed on a rod (IITC Life Sciences) rotating from 4 to 40 revolutions per minute over 5 min for 4 trials per day with a 10 min inter-trial interval. This was repeated for 3 days total. [dx.doi.org/10.17504/protocols.io.b5srq6d6](https://doi.org/10.17504/protocols.io.b5srq6d6).

DigiGait

Following a 60 min habituation, mice were placed in the DigiGait treadmill (Mouse Specifics Inc.). The treadmill ran at 22 cm/s (3- and 9-months timepoints) or 18 cm/s (18-month timepoint) with a 0° incline. 3 s of continuous movement was recorded using DigiGait Imager software.

and was then analyzed with DigiGait Analysis software. [dx.doi.org/10.17504/protocols.io.b5stq6en](https://doi.org/10.17504/protocols.io.b5stq6en).

Fear conditioning

Naïve age- and sex-matched mice were used to obtain the optimal intensity of foot shock. On Day 1 of testing, mice were placed into the Fear Conditioning apparatus (Noldus Information Technology) for 6 min during which time the mice experienced 3 tone-shock pairings (30 s tone co-terminated with a 2 s foot shock). On Day 2, mice were placed into the same Fear Conditioning apparatus for 6 min with no tone or foot shock. On Day 3, mice were placed into a different Fear Conditioning apparatus, now altered into a triangular shape with solid floor and a vanilla scent for 3 min with no tone then 3 min with the same 30 s tone but no foot shock. Freezing was measured/analyzed with EthoVision on all 3 testing days. [dx.doi.org/10.17504/protocols.io.b5suq6ew](https://doi.org/10.17504/protocols.io.b5suq6ew).

Fecal pellet composition

Mice were placed in a clean cage and their fecal pellets were collected over a 1 h period. These pellets were weighed (wet weight) then desiccated at 65°C for 19 h and reweighed (dry weight). The differences were calculated between these values to determine the water content. [dx.doi.org/10.17504/protocols.io.b5svq6e6](https://doi.org/10.17504/protocols.io.b5svq6e6).

Histology

See [Supplementary Material, Table S1](#) for a comprehensive list of antibodies used in this study.

Perfusion

Mice were sedated with 120 mg/kg Euthanyl (DIN00141704) then perfused with 1X phosphate buffered saline (PBS) then 4% paraformaldehyde (PFA). Brains were then extracted and incubated in 4% PFA for 72 h prior to a 3-step sucrose dehydration with 10, 20 and 30% sucrose (24 h each). Next, brains were flash frozen for 1 min in −40°C isopentane and cryosectioned at 40 μm. [dx.doi.org/10.17504/protocols.io.b5swq6fe](https://doi.org/10.17504/protocols.io.b5swq6fe).

Immunofluorescent staining

Cryosectioned tissue was mounted on a slide then blocked with blocking buffer (0.5% Triton X-100, 10% normal horse serum in 1X PBS) then incubated in primary antibody overnight at 4°C. Next, the sections were incubated in secondary antibody before drying at room temperature for 2 min. The sections were then covered with #1.5 coverslips and fluorescent mounting medium (Dako cat# S3023). [dx.doi.org/10.17504/protocols.io.b5s5q6g6](https://doi.org/10.17504/protocols.io.b5s5q6g6).

Quick decapitation, fixation

Mice were euthanized by isoflurane inhalation followed by decapitation and brains were quickly extracted. Brains were then submerged in 10% buffered formalin for 72 h prior to paraffin embedding and sectioning at 5 μm. [dx.doi.org/10.17504/protocols.io.b5szq6f6](https://doi.org/10.17504/protocols.io.b5szq6f6).

Diaminobenzidine (DAB) staining

Paraffin-embedded sections were deparaffinized in serial baths of xylenes and ethanol prior to a sodium citrate (10 mM sodium citrate, 0.05% Tween-20, pH 6) antigen retrieval (20 min at 95°C) and 0.9% H₂O₂ treatment (10 min). Next, sections were blocked in blocking buffer (0.1% Triton X-100, 10% normal horse serum in 1X PBS) then incubated in primary antibody overnight at 4°C. The following day the sections were incubated in secondary and tertiary antibody solution before exposure to DAB, dehydrating in baths of ethanol and xylenes and covering the tissue with Permount (Fisher Scientific cat# SP15–100) and #1.5 coverslips. [dx.doi.org/10.17504/protocols.io.b5s9q6h6](https://doi.org/10.17504/protocols.io.b5s9q6h6).

Toluidine Blue and H&E staining

Staining was performed by the Louise Pelletier Histology Core facility at the University of Ottawa on paraffin-embedded 5 μm sectioned mouse brain tissue using the Leica Autostainer XL. Briefly, the sections were deparaffinized and exposed to Toluidine Blue for 10 min. For H&E, sections were deparaffinized, exposed to hematoxylin for 7 min and eosin for 30 s. Then, sections were dehydrated and covered with #1.5 coverslips. Two serial sections for each were analyzed by an investigator blinded to the genotypes.

Stereology

Stereology was performed as previously described (19). Briefly, for each mouse, 8 cryosections were stained for TH and quantified using StereoInvestigator software (version 11.06.2). The sections (40 μm) began at the outer limit of the substantia nigra (SNc) and every 6th section was used (Bregma −2.54 to −3.88 mm). Mean section thickness was determined during counting at a frequency of 10 frames (roughly three measurements per hemisphere). The SNc was sampled by randomly translating a grid with 150 μm × 150 μm squares in the outlined SNc and applying an optical fractionator consisting of a 75 μm × 75 μm square. All stereological analyses were performed by an investigator blinded to genotypes [dx.doi.org/10.17504/protocols.io.b5ynq7ve](https://doi.org/10.17504/protocols.io.b5ynq7ve).

TH densitometry

For each mouse, 4 cryosections were stained for TH and imaged with Zeiss Axio Imager M2 and analyzed in ImageJ (version 2.3.0/1.53f). The sections (40 μm) began at the outer limit of the striatum and every 12th section was used (Bregma 1.18 to −0.22 mm). Brightfield images were captured at 10X magnification and converted to greyscale. To analyze, an investigator blinded to genotypes averaged 20 intensity measurements within the dorsolateral striatum and normalized to the average of 5 intensity measurements in the corpus callosum (background staining control). All 4 cryosections per mouse were averaged and represent 1 datapoint on the graph [dx.doi.org/10.17504/protocols.io.b5u4q6yw](https://doi.org/10.17504/protocols.io.b5u4q6yw).

Primary cortical neurons

Micro Coverglass #1.5 (Electron Microscopy Sciences) coverslips were pre-coated with poly-D-lysine (50 $\mu\text{g}/\text{ml}$) for at least overnight at 37°C, then washed with distilled water twice and air dried at room temperature for 2 h. Primary neurons were harvested from E13.5 to 15.5 pups and seeded at $\sim 1 \times 10^5$ cells per coverslip and cultured for 7 days *in vitro*. Cells were fixed in 4% PFA for 20 min, washed in 1X PBS, then blocked (10% serum +0.1% Triton X-100) for 1 h. Coverslips were stained overnight (see above immunofluorescent staining method) [dx.doi.org/10.17504/protocols.io.b5u6q6ze](https://doi.org/10.17504/protocols.io.b5u6q6ze).

Immunofluorescent imaging

Images for Figures 1 and 2 were obtained on a Zeiss AxioObserverZ1 LSM800 Confocal Microscope. Primary neuron images (Fig. 1) were obtained at 40X (oil) magnification at 8bit 1024 $\times$ 1024 resolution through a Z distance of $\sim 10 \mu\text{m}$ per image using optimal 0.27 μm spacing. Multichannel acquisition was performed using excitation/emissions: AF488 (Syn1) 493/517 nm (710V); DAPI 353/465 nm (750V); AF647 (Map2) 653/668 nm (865V) and AF594 (αSyn) 280/618 nm (700V). Images from tissue sections (Fig. 2) were obtained at 20X magnification (0.8 $\times$ zoom) at 16bit 1024 $\times$ 1024 resolution through a range of 10 μm per image using optimal 0.5 μm spacing. Multichannel acquisition was performed using excitation/emissions (laser intensity); AF488 (αSyn) 493/517 nm (630V for Cortex and DG, 620V for SNc); DAPI 353/465 nm (650V for Cortex and DG, 630V for SNc); AF647 (TH) 653/668 nm (600V). All images were processed and analyzed in ImageJ2 (version 2.3.0/1.53f) using the 3D project and Maximum Z project functions for primary neuron and tissue sections, respectively. Images for Figure 6C were obtained on a Zeiss Axio Imager M2 at 5X magnification and were analyzed in ImageJ2 (version 2.3.0/1.53f).

Nuclear αSyn quantification

Primary cortical neurons were imaged using confocal imaging and all cells in the frame of view were used to quantify the extent of αSyn nuclear localization using ImageJ2 (version 2.3.0/1.53f). First, the nuclei were delineated with DAPI then αSyn intensity was measured within the delineation. This data is shown as the level of αSyn nuclear enrichment relative to wildtype littermates. Cortex, DG and SNc were analyzed in a similar manner. Briefly, 11–35 cells were sampled per animal using ImageJ2 (version 2.3.0/1.53f), nuclei were defined with DAPI and relative intensity of nuclear αSyn versus cytoplasmic αSyn (immediately adjacent to the nucleus) was measured.

NeuN analysis

Photomicrographs were analyzed using ImageJ2 (version 2.3.0/1.53f). Briefly, NeuN positive structures (neuronal nuclei) were counted in a 200 $\mu\text{m} \times 200 \mu\text{m}$ frame (Layer II/III) or 350 $\mu\text{m} \times 350 \mu\text{m}$ frame (Layer V/VI) and then presented as NeuN+ neuron #/mm². Values represent

the average from sampling three distinct sections per brain ($n=6$ per genotype) and were analyzed by an investigator blinded to the genotypes.

Biochemistry

See [Supplementary Material, Table S1](#) for a comprehensive list of antibodies used in this study.

Serial extraction

A 0.79 mm³ punch (1 mm thick slice, 1 mm i.d. punch) of cortical brain tissue from 18-month-old mice was homogenized and resuspended in a series of increasingly stringent buffers beginning with 100 μl of TSS Buffer (140 mM NaCl, 5 mM Tris-HCl), then 100 μl TXS Buffer (140 mM NaCl, 5 mM Tris-HCl, 0.5% Triton X-100), then 100 μl SDS Buffer (140 mM NaCl, 5 mM Tris-HCl, 1% SDS), as previously described (67). Total protein levels were measured using the Pierce™ BCA Assay Kit (Thermo Fisher cat# 23225). [dx.doi.org/10.17504/protocols.io.b5vvq666](https://doi.org/10.17504/protocols.io.b5vvq666).

Western blot

Protein samples were loaded into a 12% polyacrylamide gel and subsequently transferred to a 0.2 μm nitrocellulose membrane. Membranes were blocked in a 10% milk solution then incubated in primary antibody (diluted in 2% bovine serum albumin) overnight at 4°C. Next, the membrane was incubated in a horseradish peroxidase-conjugated secondary antibody diluted in 10% milk solution. Then, the membrane was rinsed with ECL Clarity solution (Bio-Rad cat# 1705061) and imaged with a GE ImageQuant LAS 4000. [dx.doi.org/10.17504/protocols.io.b5wkq7cw](https://doi.org/10.17504/protocols.io.b5wkq7cw).

RNA extraction and real-time quantitative PCR:

RNA was extracted from mouse brain homogenate using Trizol-Chloroform extraction (Invitrogen™ User Guide: TRIzol Reagent version B.0). Briefly, mouse brains were homogenized in 3 ml of PEPI Buffer [5 mM EDTA, 1X protease inhibitor (GenDEPOT cat# P3100-020), in 1X PBS] using a dounce homogenizer. 3% of homogenate was added to 1 ml of TRIzol Reagent (Fisher Scientific cat# 15-596-026) and RNA was isolated following the user guide referenced above. cDNA was synthesized using 5X All-in-One RT Master Mix Kit (Bio Basic cat# HRT025-10). Real-time quantitative PCR was performed using Green-2-Go qPCR Master Mix (Bio Basic cat# QPCR004-S) with 25 ng cDNA per reaction and primers targeting mouse *Gapdh* (Forward: 5'-GGAGAGTGTTCCTCGTCCC-3', Reverse: 5'-ATGAAGGGTCGTTGATGGC-3'), *Hprt1* (Forward: 5'-TGATAGATCCATTCTATGACTGTAGA-3', Reverse: 5'-AAGACATTCTTCCAGTTAAAGTTGAG-3'), and *Snca* (Forward: 5'-GAAGACAGTGGAGGGAGCTG-3', Reverse: 5'-CAGGCATGTCTTCCAGGATT-3'). Reactions were run on BioRad CFX96 thermocycler (protocol: 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 60 s, then melting curve). *Snca* Ct values were standardized

to the average of *Hprt1* and *Gapdh* [dx.doi.org/10.17504/protocols.io.b5wmq7c6](https://doi.org/10.17504/protocols.io.b5wmq7c6).

Dopamine and metabolite measurement via liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)

Striatal punches (2 mm i.d., 3 mm thick section) were extracted from 18-month-old mouse brains and weighed prior to submitting to The Metabolomics Innovation Centre (TMIC). Fifty microliter of tissue extraction buffer was added to each sample tube followed by homogenization and centrifugation. Supernatant was used for LC-MS/MS analysis to get concentrations in the unit of μM . TMIC staff applied a targeted quantitative metabolomics approach to analyze the samples using a reverse-phase LC-MS/MS custom assay. This custom assay, in combination with an ABSciex 4000 QTrap (Applied Biosystems/MDS Sciex) mass spectrometer, can be used for the targeted identification and quantification of dopamine (DA), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC). The method combines the derivatization and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring (MRM) pairs. Isotope-labeled internal standards and other internal standards are used for metabolite quantification. The custom assay contains a 96 deep-well plate with a filter plate attached with sealing tape, and reagents and solvents used to prepare the plate assay. First 14 wells were used for 1 blank, 3 zero samples, 7 standards and 3 quality control samples. For all metabolites except organic acid, samples were thawed on ice and were vortexed and centrifuged at $13\,000\times g$. Ten microliter of each sample was loaded onto the center of the filter on the upper 96-well plate and dried in a stream of nitrogen. Subsequently, phenyl-isothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator. Extraction of the metabolites was then achieved by adding $300\ \mu\text{l}$ of extraction solvent. The extracts were obtained by centrifugation into the lower 96-deep well plate, followed by a dilution step with MS running solvent.

For organic acid analysis, $150\ \mu\text{l}$ of ice-cold methanol and $10\ \mu\text{l}$ of isotope-labeled internal standard mixture was added to $50\ \mu\text{l}$ of sample for overnight protein precipitation. Then it was centrifuged at $13\,000\times g$ for 20 min. Fifty microliter of supernatant was loaded into the center of the wells of a 96-deep well plate, followed by the addition of 3-nitrophenylhydrazine (NPH) reagent. After incubation for 2 h, BHT stabilizer and water were added before LC-MS injection.

Mass spectrometric analysis was performed on an ABSciex 4000 Qtrap[®] tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA). The samples were delivered to the mass spectrometer by a LC method followed by a direct injection (DI) method. Data analysis was done using Analyst 1.6.2.

TMT10plex[™] proteomics via liquid chromatography-mass spectrometry

Whole mouse cortex was dissected from 9-month-old mouse brain and peptides were isolated using the EasyPep[™] Mini MS Sample Prep Kit (ThermoFisher cat# A40006) following manufacturer instructions. These samples were labelled with the TMT10plex[™] Isobaric Label Reagent Set (ThermoFisher cat# 90406) then combined into a single tube and fractionated into 12 samples using the Pierce[™] High pH Reversed-Phase Peptide Fractionation Kit (ThermoFisher cat# 84868). Fractions 2, 3, 9, 10, 11 and 12 were combined due to low protein concentration (combined to have a consistent protein concentration with other fractions) and the 6 final fractions were submitted for liquid chromatography-mass spectrometry (LC-MS) to the Ottawa Hospital Research Institute Proteomics Core Facility. LC-MS was performed using Orbitrap Fusion Lumos mass spectrometer with UltiMate 3000 RLSC nano HPLC (Thermo Scientific). Proteowizard MS-CONVERT was used to generate peak lists for preliminary qualitative analysis using MASCOT software version 2.7.0 (Matrix Science, UK). Protein identification and quantitative analysis was performed using MaxQuant (Tyanova, Nature Protocols 2016, 11:2301). The reference proteome for peptide spectrum matching was UniProt/Mus musculus (version 2020-10-06). The MaxQuant results were exported to Scaffold Q+S (Proteome Software, USA) for further analysis and viewing. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (68) partner repository with the dataset identifier PXD032065 and [10.6019/PXD032065](https://doi.org/10.6019/PXD032065).

Statistical analyses

All statistical analyses were performed using GraphPad Prism (version 9.1.2) using the appropriate statistical test, either Student's t-test for simple comparisons or one- or two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis for multiple comparisons. The survival curve was analyzed using a log-rank Mantel-Cox test. The Mann-Whitney test with Benjamini-Hochberg correction was used with Scaffold (version 5.0.0) to analyze the TMT10plex[™] mass spectrometry dataset. Chi squared test was used to compare actual and expected Mendelian ratios of the genotypes. Statistical tests used, sample sizes, and P-values are delineated in each figure legend. All graphs plot the mean with the standard error of the mean (SEM). All figures and their quantification have been made public (doi.org/10.5281/zenodo.6082270).

Supplementary Material

Supplementary Material is available at HMG online.

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Authors' contributions

H.M.G. and M.W.C.R. conceptualized the study. H.M.G. generated the studied mouse cohorts, performed all behavioral tasks (with the help of K.M.R., Z.A.F., J.L.A.P. and S.M.C.), performed most of the histological and

biochemical assays, and imaged and analyzed most figures included in this published article. T.R.S. obtained all confocal images, performed qPCR experiments and provided significant intellectual support. K.M.R. aided in tissue acquisition and processing and provided significant intellectual support. K.H. aided in the analysis of stained tissue. J.L.A.P. and T.R.S. harvested and maintained all primary cortical neurons. M.W.C.R. designed the knockin mice, and assisted in data collection and analysis. H.M.G. and M.W.C.R. wrote the manuscript and all authors provided edits.

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Contribution: I generated AAV carrying YFP and performed Intracerebral Ventricular injections at P0 and dissections at P8 as a proof of concept to detect Cajal-Retzius cells in the mouse hippocampus. My overall contribution was ~5%.

Novel Tools and Methods

A Versatile Strategy for Genetic Manipulation of Cajal–Retzius Cells in the Adult Mouse Hippocampus

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Abstract

Cajal–Retzius (CR) cells are transient neurons with long-lasting effects on the architecture and circuitry of the neocortex and hippocampus. Contrary to the prevailing assumption that CR cells completely disappear in rodents shortly after birth, a substantial portion of these cells persist in the hippocampus throughout adulthood. The role of these surviving CR cells in the adult hippocampus is largely unknown, partly because of the paucity of suitable tools to dissect their functions in the adult versus the embryonic brain. Here, we show that genetic crosses of the $\Delta Np73$ -Cre mouse line, widely used to target CR cells, to reporter mice induce reporter expression not only in CR cells, but also progressively in postnatal dentate gyrus granule neurons. Such a lack of specificity may confound studies of CR cell function in the adult hippocampus. To overcome this, we devise a method that not only leverages the temporary CR cell-targeting specificity of the $\Delta Np73$ -Cre mice before the first postnatal week, but also capitalizes on the simplicity and effectiveness of freehand neonatal intracerebroventricular injection of adeno-associated virus. We achieve robust Cre-mediated recombination that remains largely restricted to hippocampal CR cells from early postnatal age to adulthood. We further demonstrate the utility of this method to manipulate neuronal activity of CR cells in the adult hippocampus. This versatile and scalable strategy will facilitate experiments of CR cell-specific gene knockdown and/or overexpression, lineage tracing, and neural activity modulation in the postnatal and adult brain.

Key words: adeno-associated virus; adult hippocampus; Cajal–Retzius cells; $\Delta Np73$ -Cre; genetic modification; neonatal intracerebroventricular injection

Significance Statement

High-throughput and specific tools for genetic manipulation of neuronal subtypes *in vivo* are desirable for scalable experiments and accurate data interpretation. However, limitations in available tools present a challenge for certain cell types, such as Cajal–Retzius cells, a class of transient neurons of which a portion persists in the adult brain. Highlighting the limitation of Cre-driver mouse lines because of loss of specificity in adulthood, we demonstrate the use of neonatal intracerebroventricular delivery of adeno-associated viral vectors to specifically manipulate Cajal–Retzius cells in the adult hippocampus. Our strategy offers a framework to address similar issues with experiment throughput and specificity of other neuronal subtypes.

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Introduction

Cajal–Retzius (CR) cells are a group of early-born glutamatergic neurons that populate the embryonic cortex (Squarzone et al., 2015; Causeret et al., 2021; Vilchez-Acosta et al., 2022). Originating from four distinct progenitor domains of the brain, including the ventral pallidum, the septum, the thalamic eminence, and the cortical hem, CR cells first appear at approximately embryonic day 9.5 (E9.5) in mice. They then migrate tangentially to cover the entire surface of the telencephalon. Their final destination is determined by their ontogenic origin and migration path, such that a particular brain region is predominantly occupied by CR cells from the same progenitor niche. A prominent example is the mouse hippocampus, which is populated almost exclusively by cortical hem-derived CR cells (Yoshida et al., 2006; Louvi et al., 2007). Once in residence, CR cells control cortical neuron migration via secretion of the glycoprotein reelin (Meyer et al., 2004; Yoshida et al., 2006; Tissir et al., 2009; Amelio et al., 2020; Vilchez-Acosta et al., 2022). They also regulate cortical and hippocampal circuits through their connectivity in local networks (Anstötz et al., 2022; Genescu et al., 2022; Riva et al., 2023).

CR cells can be readily identified by a few selective markers including reelin (RELN) and p73 (TRP73), of which the latter is considered to be the most specific (Anstötz and Maccaferri, 2020). Morphologically distinct from other neurons by virtue of their smaller cell body and characteristic “tadpole” shape (Anstötz et al., 2016; Anstötz and Maccaferri, 2020), CR cells can also be recognized based on their distinct locations within the brain, particularly in the marginal zone of the embryonic neocortex (Del Río et al., 1996; Gil et al., 2014; Ma et al., 2014; Elorriaga et al., 2023), as well as along the hippocampal fissure and the molecular layer of the dentate gyrus (DG; Anstötz et al.,

2016; Causeret et al., 2021; Glærum et al., 2022). Beyond protein markers and morphologic properties, an array of transgenic reporter or Cre-expressing mouse lines have been used for the study of CR cells in the developing brain (for review, see Causeret et al., 2021). For example, the $\Delta Np73$ -Cre mouse strain drives Cre expression in CR cells derived from the cortical hem, septum, and the thalamic eminence (Tissir et al., 2009), while the *Wnt3a*-Cre mouse line is restricted to cortical hem-derived CR cells (Yoshida et al., 2006). Though powerful, these genetic tools also nonspecifically label or target other cells (for review, see Causeret et al., 2021), which may cloud data interpretation.

CR cells have long been known as transient neurons as a majority of them undergo programmed cell death during development. In humans, massive CR cell death occurs around gestational weeks 23–28 (Meyer and González-Gómez, 2018a, b), with some cells persisting in layer 1 of the neocortex (Marín-Padilla, 1990; Martínez-Cerdeño et al., 2002; Martínez-Cerdeño and Clascá, 2002; Martínez-Cerdeño and Noctor, 2014) and the hippocampus (Blümcke et al., 1996, 1999). In mice, very few (~5%) CR cells persist in the adult neocortex. However, a larger portion (20–30%) of CR cells remain in the hippocampus throughout adulthood (Anstötz et al., 2016, 2018a). The persistence of CR cells in the adult hippocampus is often overlooked, which may be partly because of the presumption of their complete disappearance. Because of this assumption, most of the available genetic tools for the study of CR cells have not been thoroughly characterized regarding their cell-type specificity in the adult brain, leading to a paucity of suitable tools to specifically manipulate persistent CR cells. Moreover, as CR cells have critical roles in embryonic brain development (Causeret et al., 2021; Elorriaga et al., 2023), methods that confer specific labeling or targeting of postnatal CR cells without affecting their embryonic counterparts are especially desirable for the dissection of their contributions in the adult brain. Here, we describe a simple and versatile strategy to genetically manipulate postnatal and adult CR cells by introducing Cre-dependent adeno-associated virus (AAV) constructs to neonatal $\Delta Np73$ -Cre mice via freehand intracerebroventricular injections. This method is relatively high throughput and low cost since it does not require special surgery and injection equipment. Our strategy will facilitate experiments that enhance our understanding of the function of CR cells in the early postnatal and adult hippocampus.

Materials and Methods

Mice

$\Delta Np73$ -Cre hemizygous mice (Tissir et al., 2009) and *Wnt3a*-Cre hemizygous mice (Yoshida et al., 2006) were a gift from Alessandra Pierani (Université Paris Cité, Paris, France) and were kept as hemizygous; only hemizygous Cre mice were used throughout this study. *LSL*-*tdTomato* [B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J} (Ai9); stock #007909; Madisen et al., 2010], *LSL*-*ArchT*-EGFP [B6.Cg-Gt(ROSA)26Sor^{tm40.1(CAG-aop3/EGFP)Hze/J} (stock #021188; Daigle et al., 2018), *LSL*-*2XChETA*-*P2A*-*tdTomato* [B6;129-Gt(ROSA)26Sor^{tm1(CAG-COP4+E123T+H134R)-tdTomato)Gfng/J} (stock

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Table 1: List of genotyping PCR primers used in this study

Gene/allele target	Primers (5'–3')	PCR product size
Cre (generic)	CCGGGCTGCCACGACCAA GGCGCGGCAACACCATTTT	490 bp
<i>LSL-tdTomato</i>	AAG GGA GCT GCA GTG GAG TA CCG AAA ATC TGT GGG AAG TC GGC ATT AAA GCA GCG TAT CC CTG TTC CTG TAC GGC ATG G	Mutant = 196 bp WT = 297 bp
<i>LSL-HA-hM3D</i>	CGCCACCATGTACCCATAC GTGGTACCGTCTGGAGAGGA AAGGGAGCTGCAGTGGAGTA CCGAAAATCTGTGGGAAGTC	Transgene/mutant = 204 bp Internal positive control/WT = 297 bp
<i>LSL-ArchT</i>	AAG GGA GCT GCA GTG GAG TA CCG AAA ATC TGT GGG AAG TC ATT GCA TCG CAT TGT CTG AG CCG AAA ATC TGT GGG AAG TC	Mutant = ~300 bp WT = 297 bp
<i>LSL-ChETA</i>	CACTTGCTCTCCCAAAGTCG TACTCCGAGGCGGATCACAAAGC ACTAGTCAATAATCAATGTGACCGG	Mutant = ~300 bp WT = 452 bp
<i>ΔNP73-Cre specific</i>	ATTCTCCACCGTCAGTACG GAATGCCAACTCTCAGTCCG GTCTCTCTGAACCCCAACCA	WT = 328 bp KO = 796 bp

#017455; Ting and Feng, 2013], and *LSL-HA-hM3D* [B6N;129-Tg(CAG-CHRM3*,-mCitrine)1Ute/J, stock #026220; Zhu et al., 2016] mice were obtained from The Jackson Laboratory. Mice were group housed in a 12 h light/dark cycle, with all experiments performed during the light period. For genotyping animals between P7 and P17, tail biopsy samples were taken at the time the mice were killed, followed by tissue lysis and genotyping PCRs (Table 1). For genotyping animals older than P17, ear notch biopsy samples were taken between P14 and P18, and only animals with the desired genotypes were kept and weaned from the parents at P21. The genotypes of these mice were confirmed again by taking tail biopsy samples at the time mice were killed. Both male and female mice were used for experiments. Detailed information regarding the number and sex of animals used in each experiment is provided in Table 2. Ages are indicated where applicable. All animal procedures were performed in accordance with the animal care committee regulations of the University of Alberta and the University of Ottawa.

Adeno-associated viruses

pAAV-CAG-YFP was generated as described previously (Rousseaux et al., 2018). pAAV-hSyn-DIO-hM3D(Gq)-mCherry was a gift from Bryan Roth [the University of North Carolina School of Medicine, Viral Prep #44361-AAV8, Addgene (<http://n2t.net/addgene:44361>); RRID:Addgene_44361]. pAAV-EF1α-double floxed-hChr2(H134R)-mCherry-WPRE-HGHpA was a gift from Karl Deisseroth [Stanford University, Viral Prep #20297-AAV8, Addgene (<http://n2t.net/addgene:20297>); RRID:Addgene_20297]. Viruses were aliquoted and stored at –80°C until use. Thawed viruses were kept at 4°C and used within a week. When necessary, viruses were diluted to the desired titers using PBS.

Neonatal intracerebroventricular injections

Neonatal intracerebroventricular injections were performed as previously described with minor modifications (Kim et al., 2013). Within 6 h after birth, the newborn pups

and dam were transported to the surgery suite in their home cage. Half of the litter was removed from the cage and transferred to a biological safety cabinet. One at a time, each pup was anesthetized using hypothermia by placing the pup on a wet paper towel on ice. When the pup no longer responded to tactile stimulation, the animal was placed on a cold flat surface and the cranial surface was disinfected with a 70% ethanol wipe (catalog #326910, Becton Dickinson Canada). Using a gas-tight syringe (catalog #361025642, Hamilton) with a 32 gauge 1.25 cm needle (catalog #7762–03, Hamilton), 2 μl of AAV was injected into the lateral ventricles of each hemisphere. The injection sites were cleaned with a 70% ethanol wipe to remove any surface contamination. The pup was then placed on a 37°C heating pad until cardiac output improved, as evidenced by a bright pink skin color, and the pup regained mobility and responded to tactile stimulation. The pup was then kept warm in bedding on the heating pad until the remaining half of the litter was completed. The injected pups were then returned to the home cage together, at the same time removing the second half of the litter, thereby endeavoring to reduce stress on the dam. The remaining pups were injected with the virus as aforementioned. Once all pups were injected with the virus, the cages were then promptly returned to the housing suite. Pups were monitored daily for 1 week to ensure the absence of complications.

Clozapine-N-oxide injection

At 7 weeks of age, AAV8/hSyn-DIO-hM3D-mCherry injected mice were intraperitoneally injected with 5 mg/kg clozapine-N-oxide (CNO; 4 mg/ml stock solution; catalog #6329/10, Tocris Bioscience) or sterile saline (0.9% NaCl; catalog #JB1324, Baxter). Two hours after the intraperitoneal injection, the brain tissues were collected as described below.

Tissue preparations

Animals were deeply anesthetized via injection of sodium pentobarbital (240 mg/ml, i.p.; Euthanyl, Bimeda-

Table 2: Summary of statistical analysis

Experiment	Animal number and sex	Test	p-value	t, df	F, dfn, dfd
Figure 2B	P7: N = 6 (3 females and 3 males); P17: N = 4 (2 females and 2 males); 4W: N = 4 (2 females and 2 males); 7W: N = 7 (3 females and 4 males)				
tdT ⁺ CR cell density, hippocampal fissure		Nested one-way ANOVA	<0.0001		79.85, 3, 59
Figure 2B tdT ⁺ CR cell density, lower molecular layer		Nested one-way ANOVA	**0.0095; ****<0.0001		178.3, 3, 59
Figure 2B tdT ⁺ granule neurons, suprapyramidal blade		Nested one-way ANOVA	*0.0367; ***0.0003		6.819, 3, 59
Figure 2B tdT ⁺ granule neurons, infrapyramidal blade		Nested one-way ANOVA	**0.0024		5.054, 3, 59
Figure 2B CR cell-targeting specificity		Nested one-way ANOVA	P7 vs P17: *0.0491; P7 vs 4W: ****<0.0001 P7 vs 7W: *0.0369; 4W vs 7W: *0.0179		9.611, 3, 59
Extended Data Figure 2-1 tdT ⁺ CR cell density, neocortex		Nested one-way ANOVA	<0.0001		148.7, 3, 59
Extended Data Figure 2-3 tdT ⁺ granule neurons, suprapyramidal blade, P17		Nested t test	0.1918	t = 1.400, df = 10	1.960, 1, 10
Extended Data Figure 2-3 tdT ⁺ granule neurons, suprapyramidal blade, 4W		Nested t test	0.2669	t = 1.176, df = 10	1.382, 1, 10
Extended Data Figure 2-3 tdT ⁺ granule neurons, suprapyramidal blade, 7W		Nested t test	0.4237	t = 0.8177, df = 19	0.6686, 1, 19
Extended Data Figure 2-3 tdT ⁺ granule neurons, infrapyramidal blade, P17		Nested t test	0.212	t = 1.333, df = 10	1.777, 1, 10
Extended Data Figure 2-3 tdT ⁺ granule neurons, infrapyramidal blade, 4W		Nested t test	0.5247	t = 0.6592, df = 10	0.4345, 1, 10
Extended Data Figure 2-3 tdT ⁺ granule neurons, infrapyramidal blade, 7W		Nested t test	0.4111	t = 0.8405, df = 19	0.7064, 1, 19
Figure 3B	4W: N = 4 (2 females and 2 males); 7W: N = 7 (2 females and 2 males)				
tdT ⁺ cells, 4W		Welch's t test	0.1813		t = 1.730, df = 3.025
Figure 3B tdT ⁺ cells, 7W		Welch's t test	0.0424		t = 3.362, df = 3.060
Figure 4C	P14: n = 1 female and 1 male; 5W: n = 1 female and 3 males				
CR cell density		Nested t test	<0.0001	t = 11.02, df = 19	121.4, 1, 19
Figure 4C Granule neuron density		Nested t test	0.2771	t = 1.219, df = 5	1.487, 1, 5

(Continued)

Table 2: Continued

Experiment	Animal number and sex	Test	p-value	t, df	F, dfn, dfd
Figure 4D CR cell density (hippocampal fissure)		Nested one-way ANOVA	*0.0194; **0.0013		14.25, 2, 9
Figure 4D CR cell density (lower molecular layer)	<i>ArchT^{ΔNp73-Cre}</i> ; n = 3 males	Nested one-way ANOVA	**0.0015; ***0.0003		23.03, 2, 9
Figure 4D Granule neuron density (suprapyramidal blade)	<i>ChETA^{ΔNp73-Cre}</i> ; n = 2 females and 1 male <i>hM3Dq^{ΔNp73-Cre}</i> ; n = 1 female and 3 males	Nested one-way ANOVA	**0.0017; ****<0.0001		14.34, 2, 36
Figure 4D Granule neuron density (infrapyramidal blade)		Nested one-way ANOVA	***0.0003; ****<0.0001		21.16, 2, 36
Figure 4D CR cell targeting specificity		Nested one-way ANOVA	****<0.0001		28.05, 2, 36
Figure 4E Variation between hemispheres (<i>ArchT^{ΔNp73-Cre}</i>)	n = 3 males	Nested t test	0.1259	t = 2.545, df = 2	6.478, 1, 2
Figure 4E Variation between hemispheres (<i>hM3Dq^{ΔNp73-Cre}</i>)	n = 1 female and 3 males	Nested t test	0.1308	t = 2.486, df = 2	6.180, 1, 2
Extended Data Figure 4-2D CR cell density (Layer 1)	<i>ArchT^{ΔNp73-Cre}</i> ; n = 3 males <i>ChETA^{ΔNp73-Cre}</i> ; n = 2 females and 1 male <i>hM3Dq^{ΔNp73-Cre}</i> ; n = 1 female and 3 males	Nested one-way ANOVA	Not significant		1.863, 2, 17
Figure 5B Percentage of mCherry ⁺ CRs, hippocampal fissure	1.0 × 10 ¹¹ : N = 5 (1 female and 4 males); 1.0 × 10 ¹² : N = 4 (1 female and 3 males); 1.0 × 10 ¹³ : N = 5 (4 females and 1 male);	Nested one-way ANOVA	**0.0013; ****<0.0001		261.2, 2, 6
Figure 5B Percentage of mCherry ⁺ CRs, lower molecular layer		Nested one-way ANOVA	**0.0016; ***0.0001; ****<0.0001		152.8, 2, 6
Figure 5B Percentage of mCherry ⁺ granule neurons		Nested one-way ANOVA	10 ¹¹ vs. 10 ¹³ : 0.0032 (**); 10 ¹² vs. 10 ¹³ : 0.0025 (**)		8.613, 2, 39
Extended Data Figure 5-3C Percentage of mCherry ⁺ CRs, hippocampal fissure	1.0 × 10 ¹¹ at P14: N = 5 (2 females and 3 males); 1.0 × 10 ¹² at P14: N = 6 (1 female and 5 males); 1.0 × 10 ¹³ at P14: N = 3 (2 females and 1 male); 1.0 × 10 ¹² at 7W: N = 3 (3 females)	Nested one-way ANOVA	all not significant		0.8421, 3, 47
Extended Data Figure 5-3C Percentage of mCherry ⁺ CRs, lower molecular layer		Nested one-way ANOVA	all not significant		0.5963, 3, 47
Extended Data Figure 5-3C Number of mCherry ⁺ granule neurons		Nested one-way ANOVA	all not significant		1.443, 3, 8

(Continued)

Table 2: Continued

Experiment	Animal number and sex	Test	p-value	t, df	F, dfn, dfd
Extended Data Figure 5-3D	WT: <i>N</i> = 3 (2 females and 1 male)				
Number of mCherry ⁺ granule neurons	$\Delta Np73$ -Cre: <i>N</i> = 5 (4 females and 1 male)	Nested <i>t</i> test	0.0313		5.290, 1, 22
Figure 6B	WT: <i>n</i> = 3 (3 females)				
Percentage of mCherry ⁺ CRs, hippocampus	$\Delta Np73$ -Cre: <i>n</i> = 10 (3 females and 7 males)	Nested <i>t</i> test	<0.0001	<i>t</i> = 11.61, df = 36	134.9, 1, 36
Figure 6C	P0 to >P14: <i>n</i> = 4 (1 female and 3 males)				
Percentage of mCherry ⁺ granule neurons	P0 to >7W: <i>n</i> = 10 (3 females and 7 males)	Nested <i>t</i> test	0.0506	<i>t</i> = 2.017, df = 39	4.069, 1, 39
Figure 6D	<i>tdTΔNp73</i> -Cre: <i>n</i> = 7 (3 females and 4 males)				
Reporter-expressing granule neurons at 7W	AAV-ChR2: <i>n</i> = 10 (3 females and 7 males)	Nested one-way ANOVA	***0.0006 and 0.0007		9.297, 2, 83
Figure 6D	AAV-hM3D: <i>n</i> = 12 (5 females and 7 males)				
CR targeting specificity		Nested one-way ANOVA	***0.0003 and 0.0009		9.563, 2, 83
Figure 6E	WT, P14: <i>n</i> = 6 (1 female and 5 males)				
Percentage of mCherry ⁺ CRs, hippocampal fissure	WT, 7W: <i>n</i> = 3 (3 females)	Two-way ANOVA	Interaction: 0.2082		1.673, 1, 24
Figure 6E	$\Delta Np73$ -Cre, P14: <i>n</i> = 4 (1 female and 3 males)				
Percentage of mCherry ⁺ CRs, lower molecular layer	$\Delta Np73$ -Cre, 7W: <i>n</i> = 10 (3 females and 7 males)	Two-way ANOVA	Interaction: 0.9824		0.0004962, 1, 24
Figure 7B					
mCherry ⁺ CRs at P14, hippocampal fissure	AAV/ChR2: <i>n</i> = 4 (1 female and 3 males)	Nested <i>t</i> test	<0.0001	<i>t</i> = 5.563, df = 19	30.94, 1, 19
Figure 7B	AAV/hM3D: <i>n</i> = 6 (1 female and 5 males)				
mCherry ⁺ CRs at P14, lower molecular layer		Nested <i>t</i> test	0.0002	<i>t</i> = 4.696, df = 19	22.05, 1, 19
Figure 7D					
mCherry ⁺ CRs at 7W, hippocampal fissure	AAV/ChR2: <i>n</i> = 10 (3 females and 7 males)	Nested <i>t</i> test	0.0011	<i>t</i> = 3.422, df = 63	11.71, 1, 63
Figure 7D	AAV/hM3D: <i>n</i> = 12 (5 females and 7 males)				
mCherry ⁺ CRs at 7W, lower molecular layer		Nested <i>t</i> test	0.0072	<i>t</i> = 2.779, df = 63	7.724, 1, 63
Figure 7E	P0 to >P14: <i>n</i> = 6 (1 female and 5 males)				
mCherry ⁺ CRs at 7W, lower molecular layer	P0 to >7W: <i>n</i> = 12 (5 females and 7 males)	Nested <i>t</i> test	0.0011	<i>t</i> = 3.467, df = 52	12.02, 1, 52
Figure 7F	Wild type, P14: <i>n</i> = 8 (6 females and 2 males)				
Total CRs, hippocampal fissure	Wild type, 7W: <i>n</i> = 7 (2 females and 5 males)	Two-way ANOVA	Interaction: 0.4793		0.5136, 1, 29
Figure 7F	$\Delta Np73$ -Cre, P14: <i>n</i> = 6 (1 female and 5 males)				
Total CRs, lower molecular layer	$\Delta Np73$ -Cre, 7W: <i>n</i> = 12 (5 females and 7 males)	Two-way ANOVA	Interaction: 0.8317		0.04601, 1, 29
Extended Data Figure 7-1C					
Percentage of mCherry ⁺ CRs, hippocampal fissure		Nested <i>t</i> test	0.2784	<i>t</i> = 1.098, df = 42	1.206, 1, 42
Extended Data Figure 7-1C	P0 to >P14: <i>n</i> = 8 (6 females and 2 males)				

(Continued)

Table 2: Continued

Experiment	Animal number and sex	Test	p-value	t, df	F, dfn, dfd
Percentage of mCherry ⁺ CRs, lower molecular layer	P0 to >7W: <i>n</i> = 7 (2 females and 5 males)	No test was performed as there was no variation among replicates			
Extended Data Figure 7-1C mCherry ⁺ granule neuron density		No test was performed as there was no variation among replicates			
Extended Data Figure 7-2 Percentage of mCherry ⁺ CRs at 7W, hippocampal fissure	Female, AAV/ChR2: <i>n</i> = 3 Male, AAV/ChR2: <i>n</i> = 7	Nested one-way ANOVA	**0.0066		4.502, 3, 58
Extended Data Figure 7-2 Percentage of mCherry ⁺ CRs at 7W, lower molecular layer	Female, AAV/hM3D: <i>n</i> = 5 Male, AAV/hM3D: <i>n</i> = 7	Nested one-way ANOVA	Not significant		2.638, 3, 61
Figure 8C Percentage of mCherry ⁺ CR cells, hippocampal fissure		Nested <i>t</i> test	<0.0001	<i>t</i> = 10.30, df = 34	106.0, 1, 34
Figure 8C Percentage of mCherry ⁺ CR cells, lower molecular layer	Saline: <i>n</i> = 5 (2 females and 3 males) CNO: <i>n</i> = 7 (3 females and 4 males)	Nested <i>t</i> test	<0.0001	<i>t</i> = 10.55, df = 34	111.2, 1, 34
Figure 8C Percentage of total ⁺ CR cells, hippocampal fissure		Nested one-way ANOVA	****<0.0001		98.35, 3, 68
Figure 8C Percentage of total CR cells, lower molecular layer		Nested one-way ANOVA	****<0.0001		114.2, 3, 68
Extended Data Figure 8-1 Percentage of total ⁺ CR cells, hippocampal fissure	Saline: <i>n</i> = 5 (5 males)	Nested one-way ANOVA	Not significant		0.7748, 3, 8
Extended Data Figure 8-1 Percentage of total ⁺ CR cells, lower molecular layer	CNO: <i>n</i> = 2 (2 females)	Nested one-way ANOVA	Not significant		0.6000, 3, 36

W, Weeks; P, postnatal days; CR, Cajal-Retzius cells; WT, wild type; tdT, tdTomato; df, degree of freedom. dfn, degrees of freedom in the numerator; dfd, degrees of freedom in the denominator.

MTC), then transcardially perfused with PBS (catalog #BP399-20, Fisher Bioreagents) followed by 4% paraformaldehyde in PBS (catalog #19202, Electron Microscope Sciences). The brains were removed and postfixed in 4% paraformaldehyde overnight at 4°C, followed by sequential submersion in 15% and then 30% sucrose for 24 h at 4°C for each change. Brain tissue was cut coronally using a brain matrix, followed by cryoembedding into Optimal Cutting Temperature compound (catalog #4585, Fisher HealthCare) and subsequently frozen at −80°C. Coronal brain sections (40 μm thick) were cut using a cryostat (catalog #CM1520, Leica Microsystems) and kept at 4°C in PBS with 0.02% sodium azide (catalog #7144.8–16, BICCA) as a preservative. Coronal sections were then transferred onto Superfrost Plus microscope slides (12–550-5, Thermo Fisher Scientific) and air dried overnight. Once the slides were dry, they were used for immunofluorescence staining or stored at −80°C.

Nissl staining and immunofluorescence studies

Nissl (cresyl violet) staining was performed using standard protocols. For immunofluorescence studies, slides were postfixed in 10% phosphate buffered formalin

(catalog #SF100-4, Fisher Chemicals) for 10 min at room temperature and then washed in PBS. Antigen retrieval was performed with a citric acid-based antigen unmasking solution (catalog #H-3300, Vector Laboratories) for 30 min in a 95°C water bath. Once the slides were cooled to room temperature, they were washed twice with PBS, permeabilized with PBST (PBS + 0.3% Triton X-100; catalog #BP151-500, Fisher Bioreagents) for 20 min at room temperature and then blocked with 5% normal donkey serum (catalog #D9663-10ML, Sigma-Aldrich) diluted in PBST (blocking solution) for 20 min at room temperature. Primary antibodies were diluted in blocking solution, added onto the slides, and incubated overnight at 4°C in a humid chamber. Sections were washed three times with PBST before incubating for 2 h at room temperature in secondary antibody diluted in blocking buffer. Afterward, the slides were washed in PBST then PBS, and autofluorescence was quenched using Vector TrueVIEW autofluorescence quenching kit (catalog #SP8400, Vector Laboratories), prepared as per manufacturer instructions for 2 min at room temperature. The slides were washed, counterstained with DAPI (5 μg/ml; catalog #D3571, Thermo Fisher Scientific) for 10 min of

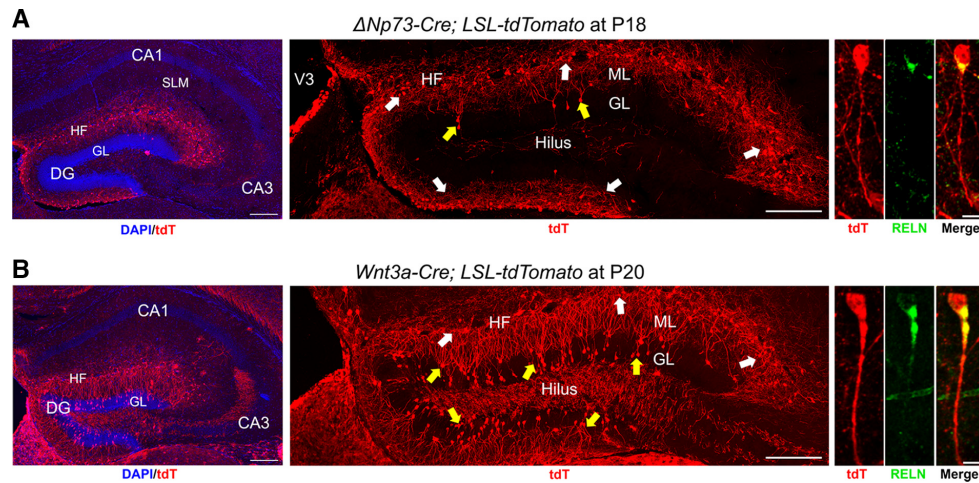


Figure 1. Characterization of tdTomato (tdT) reporter expression driven by the *ΔNp73-Cre* or the *Wnt3a-Cre* mouse line. **A, B**, Comparison of tdT reporter expression in the *ΔNp73-Cre; LSL-tdTomato* mice at P18 (**A**) and the *Wnt3a-Cre; LSL-tdTomato* mice at P20 (**B**). Left, Representative images show tdTomato expression in the hippocampus. In the hippocampus of both mouse lines, reporter expression is limited to the DG and along the hippocampal fissure (HF). Reporter expression was not detected in cornu ammonis (CA) regions. Scale bars, 200 μ m. Middle, Higher-magnification images show reporter expression in the DG. In both transgenic lines, reporter expression is readily detectable in Cajal–Retzius cells (white arrows) located along the HF and in the dentate gyrus molecular layer (ML). While cells in the granular layer (GL; yellow arrows) of the *ΔNp73-Cre; LSL-tdTomato* mice are only occasionally observed to express tdTomato, there is extensive tdTomato expression in the granular layer in the *Wnt3a-Cre; LSL-tdTomato* mice. Scale bars, 200 μ m. Right panels, Cajal–Retzius cells are identified by their unique tadpole-like morphology and the expression of RELN. Scale bars, 10 μ m. SLM, Stratum lacunosum-molecular; V3, third ventricle.

incubation at room temperature, then washed with PBS. The slides were then mounted using VECTASHIELD Vibrance Antifade Mounting Medium (catalog #H170010, Vector Laboratories) and covered with a coverslip. The slides were left to dry overnight, sealed with transparent nail polish, then further dried before being imaged with a confocal microscope.

Antibodies

The following primary antibodies were used for immunofluorescence staining: goat anti-tdTomato (1:500; catalog #AB8181-200, SIGGEN; RRID:AB_2722750); rabbit anti-TRP73 (1:500; catalog #ab40658, Abcam; RRID:AB_776999); mouse anti-RELN (1:500; catalog #MAB5364, MilliporeSigma; RRID:AB_1293544); mouse anti-RELN (1:500; catalog #ab78540, Abcam; RRID:AB_1603148); rabbit anti-CALB1 (1:500; catalog #CB38, Swant; RRID:AB_10000340); mouse anti-DCX (1:25; catalog #sc-271390, Santa Cruz Biotechnology; RRID:AB_10610966); and goat anti-EGFP/YFP (yellow fluorescent protein; 1:500; catalog #AB0020-500, SIGGEN; RRID:AB_2333100). The secondary antibodies used were as follows: donkey anti-goat Alexa Fluor 555 (1:1000; catalog #A21432, Thermo Fisher Scientific; RRID:AB_2535853); donkey anti-rabbit Alexa Fluor 488 (1:1000; catalog #A21206, Thermo Fisher Scientific; RRID:AB_2535792); and donkey anti-mouse Alexa Fluor 647 (1:1000; catalog #A31571, Thermo Fisher Scientific; RRID:AB_162542).

Confocal microscopy

Immunofluorescent images were taken using a laser-scanning confocal microscope (model LSM 700, Zeiss). For adult (>5 weeks old) mouse brains, three coronal sections from each animal spanning the dorsal dentate gyrus at bregma -1.46 , -1.94 , and -2.46 mm were selected for imaging. For younger mice, comparable anatomic sections were chosen for imaging. Tiled and z-stacked images were acquired for each animal.

Data analyses and statistical method

Cell counting and area measurements were performed using Fiji ImageJ software (version 1.53; Schindelin et al., 2012). The hippocampal fissure area was defined as 60 μ m above and below the hippocampal fissure, as previously described (Pahle et al., 2020). CR cells positive for TRP73 expression were counted along the entire hippocampal fissure or the entire molecular layer ventral to the infrapyramidal blade of the dentate gyrus (lower molecular blade) from at least three sections per animal. Cell densities were normalized to the length of the hippocampal fissure and/or the length of the lower molecular layer. The variation index was calculated using the difference of cell densities between the two hemispheres divided by the sum of the densities. Animals without any tdTomato-expressing granule neurons (three of seven mice at 7 weeks of age) were excluded from the granule neuron variation index calculation as they resulted in an invalid formula for the variation index.

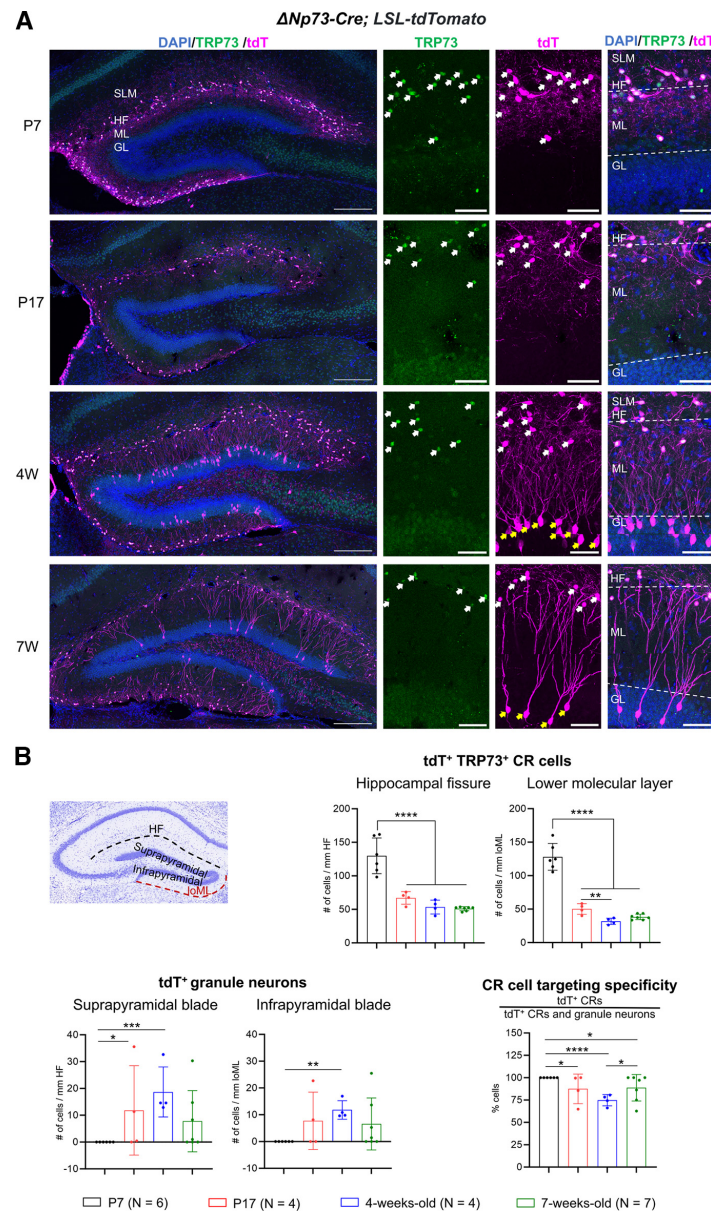


Figure 2. Reporter expression in the $\Delta Np73$ -Cre; LSL-tdTomato mice. **A**, Left, Representative confocal images show tdTomato (tdT) reporter expression in the $\Delta Np73$ -Cre; LSL-tdTomato mice at P7, P17, 4 weeks (4W), and 7W. Right panels, High-magnification images are shown with coimmunostaining for tdTomato and the Cajal-Retzius (CR) cell marker TRP73. TRP73⁺ CR cells expressing tdTomato are marked with white arrows, while TRP73⁺ granule neurons of the dentate gyrus are marked with yellow arrows. SLM, Stratum lacunosum-molecular; HF, hippocampal fissure; GL, granular layer; ML, molecular layer. Scale bars: whole dentate gyrus, 200 μ m; higher magnification, 20 μ m. **B**, Left, An image of a Nissl-stained mouse brain coronal section depicts the areas of interest. loML, Lower ML (i.e., ML of the infrapyramidal blade). Right, Quantification of tdTomato⁺ CR cells and granule neurons, including quantification of CR cell-targeting specificity. Data are presented as scatter plots with all data points shown and error bars representing $\pm$ SD. Each data point is an individual animal, whereby three sections were measured for each animal. Statistical analyses

continued

were performed using nested one-way ANOVA with Tukey's *post hoc* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The data that illustrate reporter expression in the neocortex are shown in Extended Data Figure 2-1. The images that demonstrate granule neuron identity are shown in Extended Data Figure 2-2. Analyses of the effect of sex on tdTomato-expressing granule neurons are provided in Extended Data Figure 2-3.

calculation. Statistical analyses were performed using GraphPad Prism [version 9.4.1; GraphPad Software (www.graphpad.com)]. Detailed statistics for all analyses in the article are presented in Table 2.

Results

Neither *Wnt3a-Cre* nor $\Delta Np73-Cre$ lines are specific to Cajal–Retzius cells in the hippocampus by the third postnatal week

Several Cre-driver transgenic mouse lines have been developed to study CR cells in the developing brain (Causseret et al., 2021). However, few studies have pursued the role of CR cells within the adult brain. To achieve this, we first set out to identify a transgenic mouse line that will allow us to specifically manipulate CR cells in the hippocampus. We assessed two popular mouse lines used to drive Cre expression in cortical hem-derived CR cells, the $\Delta Np73-Cre$ line (Tissir et al., 2009) and the *Wnt3a-Cre* line (Yoshida et al., 2006), by crossing them to the Cre-dependent *LSL-tdTomato* reporter mice. At P18, the $\Delta Np73-Cre$ line demonstrated high specificity in the hippocampus with tdTomato reporter expression mostly restricted to CR cells, which were characterized by their unique tadpole-like morphology, reelin expression, and localization along the hippocampal fissure and in the molecular layer of the dentate gyrus (Fig. 1A). In contrast, the *Wnt3a-Cre* line drove broad tdTomato expression outside of the CR cell domain, in what were likely DG granule neurons (Fig. 1B), consistent with previous reports (Quattrocchio and Maccaferri, 2014; Anstötz et al., 2018a) and the expression of *Wnt3* in the postnatal hippocampal neurogenic niche (Lie et al., 2005). As such, we opted to use the $\Delta Np73-Cre$ line for the remainder of this study.

In the hippocampus of the $\Delta Np73-Cre$; *LSL-tdTomato* mice, we occasionally observed non-CR cells labeled in the DG granular layer (Fig. 1A, yellow arrows). This prompted us to further characterize the extent of reporter expression in the hippocampus of these mice throughout postnatal development. At P7, tdTomato reporter expression was seen exclusively in CR cells in the DG (Fig. 2A). From P7 to P21, CR cells in the hippocampus undergo massive cell death, with ~50% of the entire population being eliminated. CR cell number continues to decline until ~5 weeks of age then stabilizes, leaving ~20–30% of CR cells persisting throughout adulthood (Anstötz et al., 2018a). In agreement with this, we observed a drastic reduction of tdTomato-expressing CR cells along the hippocampal fissure and in the molecular layer from P7 to P17, which then stabilized by 4 weeks of age (Fig. 2A,B), suggesting that the $\Delta Np73-Cre$ and the *LSL-tdTomato* alleles do not overtly alter the time course of developmental programmed cell death of CR cells. In the neocortex, reporter expression was restricted to CR cells in layer 1, and CR cell density in 7-week-old mice was reduced to ~5% of that in P7 mice (Extended Data Fig. 2-1).

Coincidental with the postnatal pruning of the CR cell population, the DG undergoes protracted development. The DG develops from E13 to P15 in mice, with continuous neurogenesis throughout adulthood thereafter (Yu et al., 2014). At P7, when the DG is still developing, we did not find any cells in the granular layer with reporter expression (Fig. 2A,B). At P17, shortly after DG morphologic maturation is completed and when mature granule neurons begin to emerge (Hochgerner et al., 2018), reporter expression was detected in some non-CR cells in the DG granular layer. To determine the identity of these tdTomato-expressing non-CR cells, we immunostained for doublecortin (DCX) and calbindin (CALB1), which mark immature and mature DG granule neurons, respectively (Hourigan et al., 2021). We found them to be DCX[−] but CALB1⁺, indicating these were mature granule neurons (Extended Data Fig. 2-2). There is a general trend that the numbers of these tdTomato-expressing non-CR cells increase after P17. By 4 weeks of age, we observed a significant increase in tdTomato-expressing granule neurons in the granular layer of the suprapyramidal and infrapyramidal blades of the dentate gyrus, as well as a concomitant decrease in CR cell-targeting specificity (Fig. 2B). Of note, reporter expression in granule neurons between different mice varied substantially (Figs. 2B, 3A), although these animals were housed under the same conditions and were handled and genotyped in a similar way. These animals were from different litters, and we did not detect any sex effects on the variability (Extended Data Fig. 2-3). We also noted significant variation between the DGs of both hemispheres within the same mouse (Fig. 3B). We further quantified this variation using the variation index, which is the difference of cell densities between the two hemispheres divided by the sum of the densities. If the two hemispheres have similar densities of cells, then the variation index would be near zero. Our analysis showed that there was limited between-hemisphere variation for CR cells in $\Delta Np73-Cre$; *LSL-tdTomato* mice, whereas the variation indexes for granule neurons were significantly higher (Fig. 3B). This suggests that the $\Delta Np73-Cre$ allele may drive recombination in mature granule neurons because of individual differences (e.g., neurobehavior, physical activity, and other individual experiences), rather than because of transient Cre expression in a regulated developmental program. Overall, our genetic crosses indicate that, while both *Wnt3a-Cre* and $\Delta Np73-Cre$ lines induce robust reporter expression in CR cells, they also lead to additional recombination in postnatal DG granule neurons by the third postnatal week.

The $\Delta Np73-Cre$ allele drives recombination in dentate gyrus granule neurons in the adult hippocampus of multiple reporter lines

While the *Wnt3a-Cre* allele has been shown to induce recombination in non-CR cells in the DG (Quattrocchio and Maccaferri, 2014; Anstötz et al., 2018a), the surprising

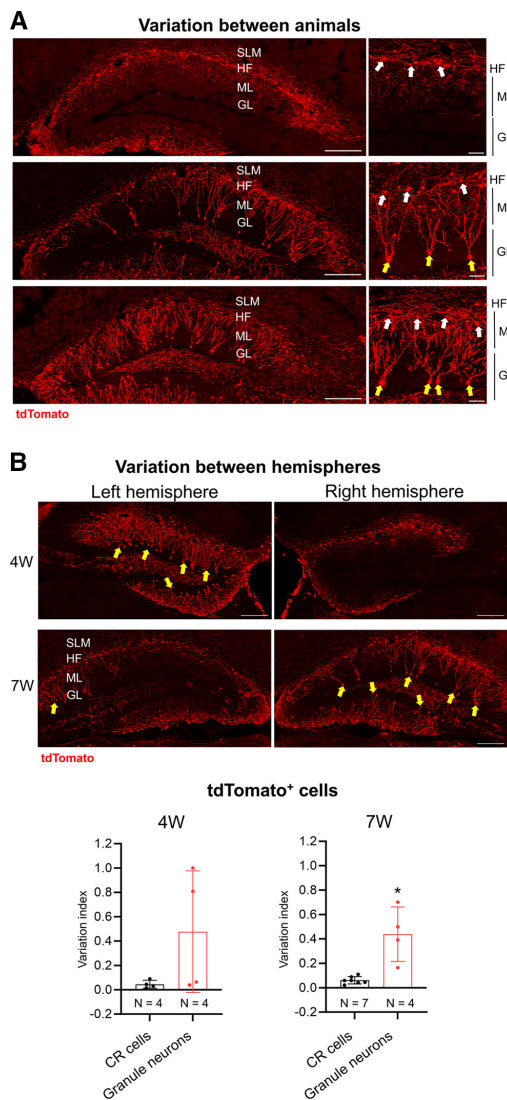


Figure 3. Variable expression of tdTomato reporter in 7-week-old $\Delta Np73$ -Cre; *LSL-tdTomato* mice. **A**, Left, Representative confocal images show variable tdTomato expression in the DG between different animals at 7 weeks of age. Each image represents an individual animal. Scale bars, 200 μ m. Right, Higher-magnification images show CR cells (white arrows) and dentate gyrus granule neurons (yellow arrows). Scale bars, 50 μ m. **B**, Representative confocal images show variable tdTomato expression between the two hemispheres of a 4-week-old and a 7-week-old animal. Yellow arrows point to granule neurons. Quantification of the variation indexes (the difference of cell densities between the two hemispheres divided by the sum of the cell densities) is shown at the bottom. Between the two hemispheres of 7-week-old animals, tdTomato expression in

continued

granule neurons is significantly more variable than that in CR cells. Data are presented as scatter plots with all data points shown and error bars representing $\pm$ SD. Each data point is an individual animal, whereby three sections were measured for each animal. Statistical analyses were performed using Welch's *t* test. $^*p < 0.05$. SLM, Stratum lacunosum-moleculare; HF, hippocampal fissure; ML, molecular layer; GL, granular layer.

degree of recombination in granule neurons by the $\Delta Np73$ -Cre allele has not been previously appreciated. We asked whether these recombination events could be because of the *LSL-tdTomato* reporter undergoing recombination in the absence of the Cre recombinase. However, we did not observe any tdTomato expression in granule neurons in the reporter-only mice (without the Cre allele; Extended Data Fig. 4-1). To further test this in a more rigorous way, we crossed the $\Delta Np73$ -Cre transgenic mice to two different reporter lines, *LSL-ArchT-EGFP* and *LSL-2XChETA-P2A-tdTomato*, expressing inhibitory and excitatory opsins, respectively, in a Cre-dependent manner. Adult (10 weeks old) $\Delta Np73$ -Cre; *LSL-ArchT-EGFP* (*ArchT* ^{$\Delta Np73$ -Cre}, for short) mice exhibited ArchT-EGFP fusion protein expression in CR cells, which were marked by the CR cell-specific protein TRP73. However, they also showed widespread EGFP expression in DG granule neurons (Fig. 4A). Similarly, we found reporter expression in both TRP73⁺ CR cells and TRP73⁻ DG granule neurons in adult $\Delta Np73$ -Cre; *LSL-2XChETA-P2A-tdTomato* (*ChETA* ^{$\Delta Np73$ -Cre}, for short) mice (Fig. 4B). All three of the aforementioned reporter alleles—the *LSL-tdTomato*, the *LSL-ArchT-EGFP*, and the *LSL-2XChETA-P2A-tdTomato*—were inserted into the *ROSA26* locus on chromosome 6, which is widely used to harbor reporter constructs because of its ubiquitous expression (Soriano, 1999). As genomic context may influence reporter expression, we next crossed the $\Delta Np73$ -Cre mice to the *LSL-HA-hM3D* mice, in which the Cre-dependent chemogenetic receptor (*hM3D*) cassette was integrated into chromosome 14 as a transgene (<https://www.jax.org/strain/026220>). At P14, we observed robust and specific reporter expression in neocortical and hippocampal CR cells in the $\Delta Np73$ -Cre; *LSL-HA-hM3D* (*hM3D* ^{$\Delta Np73$ -Cre}, for short) mice (Fig. 4C, Extended Data Fig. 4-2C). At this age, a low degree of recombination (<1 cell/mm) was seen in granule neurons, which increased to ~6 cells/mm at 5 weeks of age (Fig. 4C), which is consistent with our findings in the $\Delta Np73$ -Cre; *LSL-tdTomato* mice (Fig. 2).

We further compared the on-target and off-target recombination events of the three reporter lines under the $\Delta Np73$ -Cre driver. To this end, we quantified the number of reporter-expressing CR cells along the hippocampal fissure and in the molecular layer of the DG infrapyramidal blade, as well as the number of reporter-expressing DG granule neurons in the suprapyramidal and infrapyramidal blades. The numbers of CR cells and granule neurons were similar between the *ArchT* ^{$\Delta Np73$ -Cre} and the *hM3D* ^{$\Delta Np73$ -Cre} mice (Fig. 4D). In contrast, the *ChETA* ^{$\Delta Np73$ -Cre} mice had a lower number of labeled CRs but a higher number of granule neurons, resulting in lower CR cell-targeting specificity. While all three reporter lines had a similar number of reporter-expressing CR cells in the adult neocortex (Extended Data Fig. 4-2D),

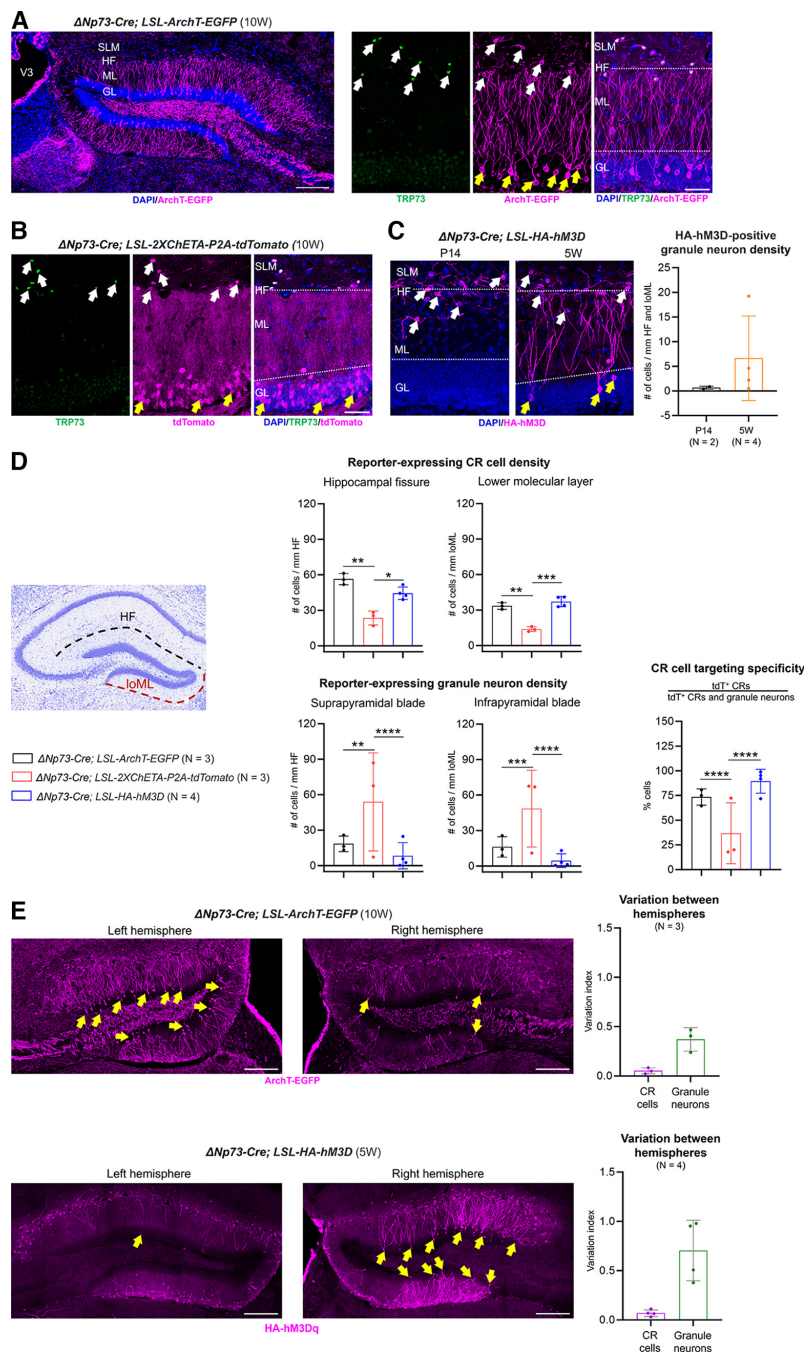


Figure 4. The $\Delta Np73$ -Cre allele drives reporter expression in Cajal–Retzius cells and adult dentate gyrus granule neurons in different reporter lines. **A**, Left, Overview of ArchT-EGFP expression in the DG of 10-week-old $\Delta Np73$ -Cre; LSL-ArchT-EGFP mice. Note the

continued

extensive ArchT-EGFP fusion protein expression in the dendrites and axons of DG granule neurons. Scale bar, 200 μ m. Right, Representative high-magnification images. ArchT-EGFP expression is found in the plasma membrane of TRP73⁺ CR cells (white arrows) and TRP73⁻ DG granule neurons (yellow arrows). Scale bar, 50 μ m. **B**, tdTomato reporter expression in the DG of 10-week-old (10W) Δ Np73-Cre; LSL-2XChETA-P2A-tdTomato mice. In these mice, ChETA and tdTomato are expressed separately and not as fusion proteins. tdTomato expression is found in the cytoplasm of TRP73⁺ CR cells (white arrows) and TRP73⁻ DG granule neurons (yellow arrows). Scale bar, 50 μ m. **C**, HA-hM3D reporter expression in the dentate gyrus of the Δ Np73-Cre; LSL-HA-hM3D mice. At P14, reporter expression is restricted to CR cells (white arrows). By 5 weeks of age, reporter expression is also observed in DG granule neurons (yellow arrows). Scale bar, 50 μ m. Quantification of reporter-expressing granule neuron density is shown to the right. Cell densities were normalized to the length of the hippocampal fissure (HF) and the molecular layer of the DG infrapyramidal blade (IoML). **D**, Quantification of reporter-expressing CR cell and granule neuron densities, as well as CR cell-targeting specificity in the three mouse lines. **E**, Representative confocal images of reporter expression in the two hemispheres of a Δ Np73-Cre; LSL-ArchT-EGFP mouse (top) and a Δ Np73-Cre; LSL-HA-hM3D mouse (bottom). Yellow arrows indicate reporter-expressing DG granule neurons. Scale bars, 200 μ m. Quantification of variation indexes (the difference of cell densities between the two hemispheres divided by the sum of the cell densities) are shown to the right. V3, Third ventricle; SLM, stratum lacunosum-moleculare; GL, granular layer. Data are presented as scatter plots with all data points shown and error bars representing $\pm$ SD. Each data point is an individual animal, whereby three sections were measured for each animal. Statistical analyses were performed using nested *t* test or nested one-way ANOVA with Tukey's *post hoc* test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. The images that show the lack of reporter expression in the hippocampus of the LSL-tdTomato mice are provided in Extended Data Figure 4-1. The data that demonstrate reporter expression in the neocortex are shown in Extended Data Figure 4-2.

recombination in the ArchT ^{Δ Np73-Cre} and the hM3D ^{Δ Np73-Cre} mice was restricted to CR cells in neocortex layer 1, while additional recombination was observed in non-CR cells in layer 1 in the ChETA ^{Δ Np73-Cre} mice (Extended Data Fig. 4-2B).

Similar to our findings with the Δ Np73-Cre; LSL-tdTomato mice (Fig. 3), the extent of Cre-mediated recombination in DG granule neurons was highly variable between individual ChETA ^{Δ Np73-Cre} mice (Fig. 4D, note the large variation among the three animals). Although this between-mice variation was less in the ArchT ^{Δ Np73-Cre} and the hM3D ^{Δ Np73-Cre} lines, recombination events in DG granule neurons varied substantially between the two hemispheres within a single mouse in these two lines (Fig. 4E). When we quantified this using a variation index, we found that the between-hemisphere variation indexes for CR cells were close to zero in both the ArchT ^{Δ Np73-Cre} and the hM3D ^{Δ Np73-Cre} mice, whereas the variation indexes for granule neurons were larger and more variable (Fig. 4E). Collectively, our data demonstrate that, while the degree and variability of recombination in granule neurons differ among reporter lines, recombination events in postnatal DG granule neurons driven by the Δ Np73-Cre allele is a consistent phenomenon among different reporter lines.

Neonatal intraventricular injection of adeno-associated virus efficiently transduces Cajal–Retzius cells

The unexpected recombination pattern driven by the Δ Np73-Cre could confound the interpretation of experiments involving genetic crosses to floxed alleles. We therefore set out to develop a strategy for efficient and specific genetic manipulation of CR cells. This was guided by our observation that, before the first two postnatal weeks, recombination by the Δ Np73-Cre allele in the hippocampus was highly specific to CR cells (Figs. 2A, 4C). Imposing additional specificity that limits recombination in DG neurons that are born after P14 could be a viable approach. Fortunately, neonatal (P0) intracerebroventricular

injection of AAV has been shown to produce very little viral-mediated expression in DG granule neurons, especially those generated after P14 (Kim et al., 2013, 2014). This free-hand injection method is fast and easy to use. By opting for a serotype such as AAV8, it results in brain-wide transduction (Kim et al., 2013), which is particularly desirable for CR cells as they are distributed throughout the hippocampus. We first conducted a pilot experiment by injecting AAV8 carrying a viral construct encoding YFP under the CAG promoter (AAV8/CAG-YFP) into P0 wild-type mice and analyzed them at P8 (Extended Data Fig. 5-1A). We found that the AAV8 serotype effectively transduced TRP73⁺ CR cells (Extended Data Fig. 5-1B, yellow arrows). We next asked whether we could use neonatal intracerebroventricular injection of Cre-dependent AAV8 constructs to impose additional temporal specificity in the Δ Np73-Cre mice. To test this, we injected P0 Δ Np73-Cre pups intraventricularly with AAV8 carrying a Cre-dependent expression construct for the ChR2-mCherry fusion protein driven by the ubiquitous EF1 α promoter [AAV8/EF1 α -DIO-ChR2-mCherry (or AAV/DIO-ChR2 for short); Fig. 5A]. As the viral titer may impact the efficiency and specificity of transduction, we determined the optimal titer by assessing transduction efficiency and specificity at 1.0×10^{11} , 1.0×10^{12} , and 1.0×10^{13} genome copies (GC)/ml. At 14 d post injection (which is also P14), we found very few CR cells with mCherry expression in mice injected with 1.0×10^{11} GC/ml virus (Fig. 5A,B), indicating that the viral titer was too low to render detectable transduction. In contrast, Δ Np73-Cre mice injected with 1.0×10^{12} GC/ml AAV8 showed robust reporter expression in ~45% of CR cells along the hippocampal fissure and ~40% of CR cells in the molecular layer of the infrapyramidal blade of the dentate gyrus (lower molecular layer). The identity of mCherry-expressing CR cells was further confirmed by immunostaining for the CR cell markers TRP73 and RELN (Fig. 5C). With this titer, viral transduction was restricted to CR cells in the hippocampus, as we did not observe reporter expression in any other hippocampal neurons such as DG granule neurons (Fig. 5A,B, Extended Data Fig. 5-2A). Slightly but significantly more robust CR cell

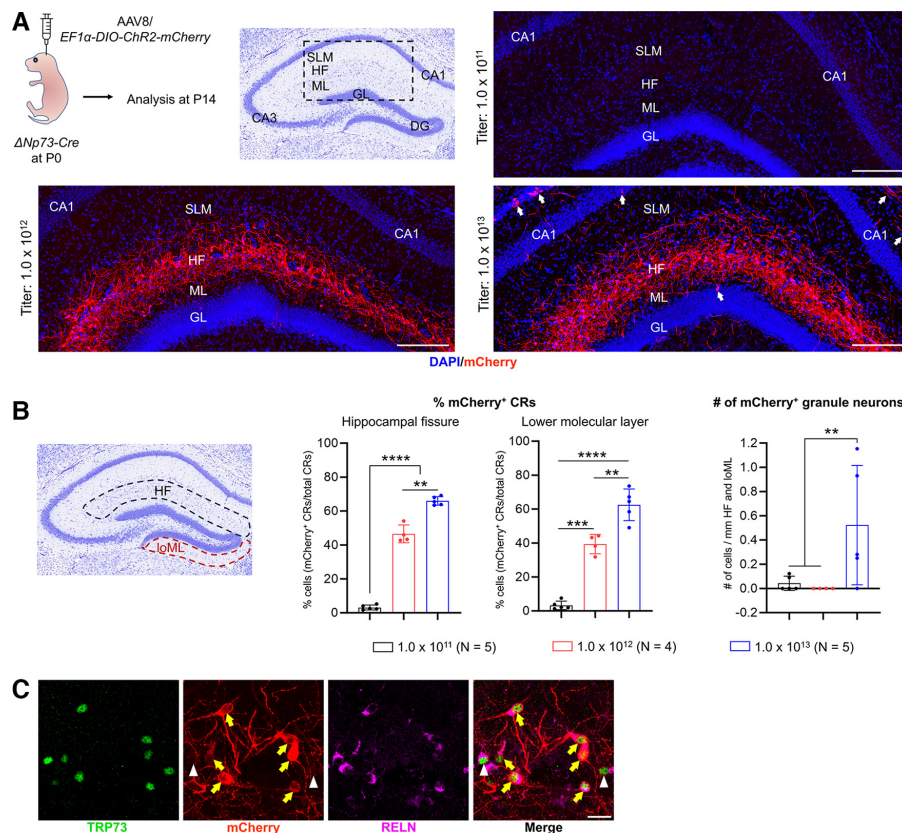


Figure 5. Neonatal intraventricular injection of adeno-associated virus induces efficient recombination events in hippocampal Cajal-Retzius cells in the $\Delta Np73$ -Cre mice. **A**, Schematic shows the experimental procedure and brain region of interest. The lateral ventricles of P0 $\Delta Np73$ -Cre mouse pups were freehand injected with different titers of AAV serotype 8 carrying a Cre-dependent expression construct for the ChR2-mCherry fusion protein under the $EF1\alpha$ promoter (AAV8/ $EF1\alpha$ -DIO-ChR2-mCherry). Injected mice were analyzed at P14. Three representative confocal images show the hippocampal fissure (HF) area after intraventricular injection of three titers of the AAV8 for comparison of transduction efficiency. Viral-mediated, Cre-dependent transgene expression is visualized by immunostaining for mCherry. Note the lack of transduction using the 1.0×10^{11} viral titer. Cre-independent spontaneous recombination events were observed in some cortical and hippocampal neurons in mice injected with the 1.0×10^{13} titer (white arrows). Scale bars, 200 μ m. **B**, Quantification of viral transduction efficiency in CR cells and recombination events in granule neurons. Data are presented as scatter plots with all data points shown and error bars representing $\pm$ SD. Each data point is an individual animal, whereby three sections were measured for each animal. The total number of CR cells per animal analyzed along the hippocampal fissure of different animals were as follows: 1.0×10^{11} titer, 90–174 cells; 1.0×10^{12} titer, 82–152 cells; 1.0×10^{13} titer, 108–188 cells. The total number of CR cells per animal analyzed in the molecular layer of the infrapyramidal blade of different animals were as follows: 1.0×10^{11} titer, 47–83 cells; 1.0×10^{12} titer, 39–82 cells; 1.0×10^{13} titer, 42–77 cells. Statistical analyses were performed using nested one-way ANOVA with Tukey's *post hoc* test. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. **C**, Immunostaining for TRP73, mCherry, and RELN in P14 1.0×10^{12} GC/ml virus-injected tissue confirmed that mCherry-expressing cells (yellow arrows) are CR cells. Some CR cells are not transduced as they do not express mCherry (white arrowheads). Scale bar, 20 μ m. GL, Granular layer; ML, molecular layer; SLM, stratum lacunosum-moleculare; CA1, Cornu Ammonis 1; CA3, Cornu Ammonis 3; loML, lower molecular layer (i.e., molecular layer of the infrapyramidal blade of the dentate gyrus). The images that illustrate the result using AAV/CAG-YFP are shown in Extended Data Figure 5-1. The images that show the specificity of our neonatal AAV injection approach in the hippocampus are provided in Extended Data Figure 5-2. The data that demonstrate the absence of Cre-mediated recombination in the wild-type mice are shown in Extended Data Figure 5-3.

transduction was observed along the hippocampal fissure (~65%) and in the lower molecular layer (~63%) in $\Delta Np73$ -Cre pups injected with 1.0×10^{13} GC/ml virus. However, at such a high titer, we began to see occasional reporter

expression in DG granule neurons (~1–3 cells/section; Fig. 5B, cell density calculation) and in CA1 neurons as well as other cortical neurons (Fig. 5A, white arrows). This could be because of Cre-independent spontaneous recombination of

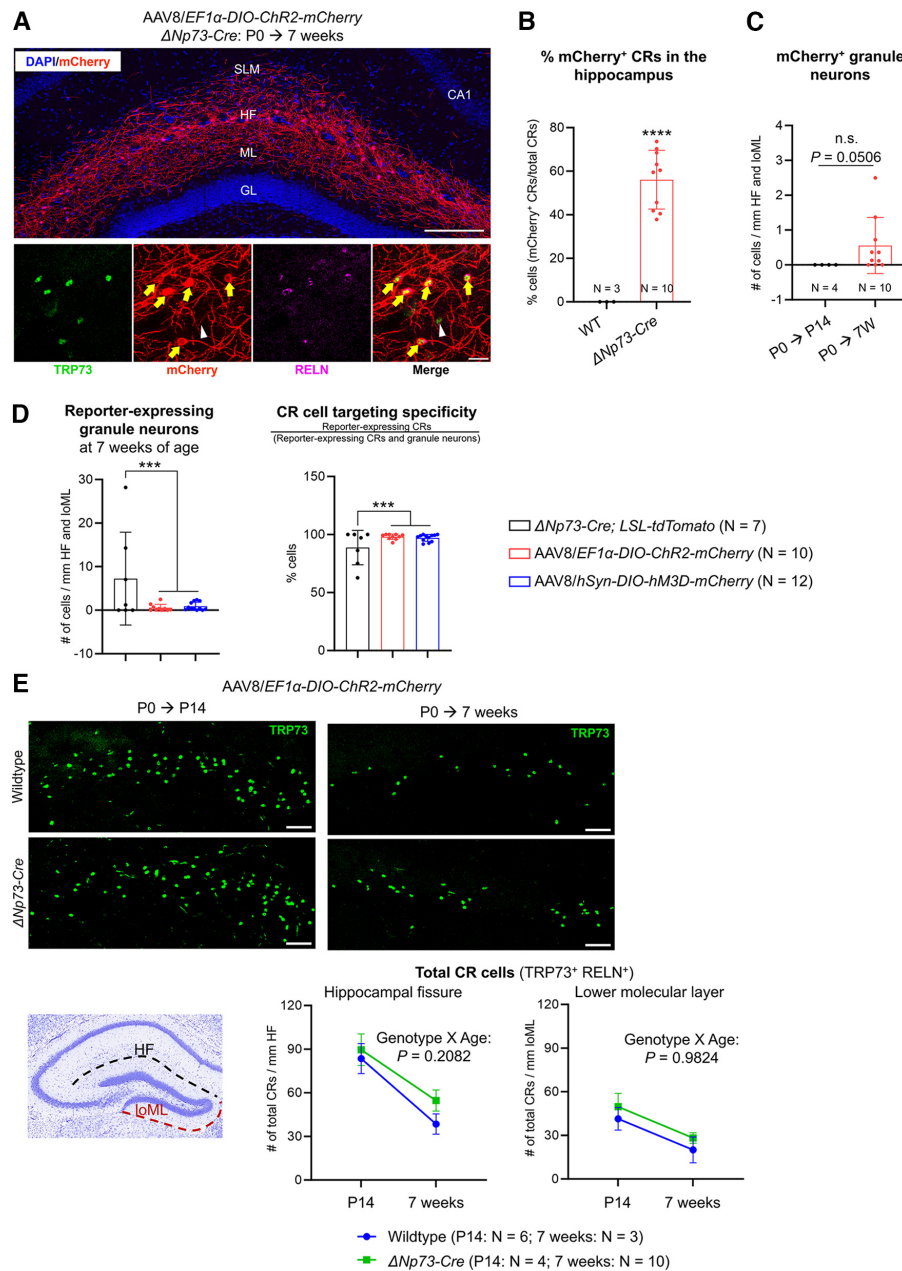


Figure 6. Neonatal intraventricular injection of adeno-associated virus results in lasting and highly specific transgene expression in Cajal–Retzius cells. **A**, Representative images of a Δ Np73-Cre mouse hippocampus 7 weeks post-neonatal (P0) injection with AAV8/EF1 α -DIO-ChR2-mCherry. Coimmunostaining with TRP73 and RELN indicates CR cells continue to express mCherry 7 weeks following the injection (yellow arrows). Some CR cells do not express mCherry (white arrowhead) because they were not transduced by the virus. Scale bars: top, 200 μ m; bottom, 20 μ m. **B**, Quantification of AAV-transduced CR cells (mCherry⁺ TRP73⁺) in 7-week-old wild-type (WT) and Δ Np73-Cre mice. **C**, Comparison of the number of mCherry⁺ granule neurons in P14 and 7-week-old

continued

$\Delta Np73$ -Cre mice neonatally injected with AAV8/EF1a-DIO-ChR2-mCherry. **D**, Comparison of the number of recombination events in granule neurons and CR cell-targeting specificity of 7-week-old $\Delta Np73$ -Cre; *LSL-tdTomato* mice, $\Delta Np73$ -Cre mice neonatally injected with AAV8/EF1a-DIO-ChR2-mCherry, and $\Delta Np73$ -Cre mice neonatally injected with AAV8/hSyn-DIO-hM3D-mCherry. **E**, Representative images of the hippocampal fissure areas of P14 and 7-week-old wild-type and $\Delta Np73$ -Cre transgenic mice injected neonatally with AAV8/EF1a-DIO-ChR2-mCherry. Scale bars, 20 μ m. Quantification of the densities of TRP73⁺ CR cells are shown at the bottom. SLM, Stratum lacunosum-moleculare; HF, hippocampal fissure; ML, molecular layer; GL, granular layer; CA1, Cornu Ammonis 1; loML, lower molecular layer (i.e., molecular layer of the infrapyramidal blade of the dentate gyrus). Data are presented as scatter plots in **B**, **C**, and **D** with all data points shown and error bars representing $\pm$ SD, and statistical analyses were performed using nested one-way ANOVA with Tukey's *post hoc* test. Each data point is an individual animal, whereby three sections were measured for each animal. Data are presented as summary data in **E** with mean $\pm$ SD, and statistical analyses were performed using two-way ANOVA with Sidak test to correct for multiple comparisons. *** $p < 0.001$; **** $p < 0.0001$; n.s., not significant.

the viral vector (Fischer et al., 2019; Botterill et al., 2021). To test this, we injected wild-type mice with AAV/DIO-ChR2 at a titer of 1.0×10^{13} GC/ml and analyzed them at P14. Our data show that recombination events in the wild-type mice, which were Cre independent, were significantly fewer than those in the $\Delta Np73$ -Cre mice, which could be both Cre dependent and Cre independent (Extended Data Fig. 5-3A, C,D). This suggests that both Cre-dependent and Cre-independent recombination events may occur when viral titers are used at 1.0×10^{13} GC/ml. Additionally, two of the five pups injected with 1.0×10^{13} GC/ml virus were runty and reached humane endpoint by P14, while animals injected with lower titers appeared healthy until at least 7 weeks of age. Therefore, we opted to use the 1.0×10^{12} GC/ml titer of the virus for the remainder of the study. Overall, our data demonstrate the specificity and effectiveness of neonatal intracerebroventricular injections of AAVs in transducing CR cells in the hippocampus.

Neonatal intraventricular injection of Cre-dependent adeno-associated virus confers specificity in Cajal–Retzius cells in the adult hippocampus

We next assessed whether neonatal intracerebroventricular injections of Cre-dependent AAV could impose sufficient temporal specificity that overcomes excessive recombination in postnatal DG granule neurons in the $\Delta Np73$ -Cre mice. To this end, animals were injected intraventricularly at P0 with 1.0×10^{12} GC/ml AAV8/DIO-ChR2 and allowed to age to 7 weeks of age. Our analysis showed that ~56% of CR cells expressed the mCherry reporter at this age (Fig. 6A,B, Extended Data Fig. 5-3B,C), confirming robust viral transduction. Importantly, recombination events in DG granule neurons were limited: of the 10 animals that we examined, 3 exhibited no recombination in granule neurons, while the other 7 animals had a limited number of recombined granule neurons (Fig. 6C). Compared with similarly injected mice at P14, there was a trend toward more recombined granule neurons at 7 weeks of age, but this did not reach significance. Nonetheless, in 7-week-old $\Delta Np73$ -Cre mice injected with AAV8/DIO-ChR2 at P0, the number of granule neurons with recombination events is significantly less than that in 7-week-old $\Delta Np73$ -Cre; *LSL-tdTomato* mice, resulting in higher CR cell-targeting specificity (Fig. 6D). This demonstrates that our approach of neonatal intracerebroventricular AAV injection into the $\Delta Np73$ -Cre pups effectively limits recombination events in granule neurons in adult mice.

CR cells are known to undergo massive cell death in early postnatal weeks (Causeret et al., 2021). We next assessed whether viral transduction or transgene expression would alter the developmental programmed cell death of CR cells by comparing wild-type and $\Delta Np73$ -Cre mice injected with the AAV. We analyzed the numbers of TRP73⁺ RELN⁺ CR cells (total CR cells) along the hippocampal fissure and in the lower molecular layer at P14, during the time window of massive CR cell death and at 7 weeks of age, when CR cell death has subsided (Anstötz et al., 2018a). We did not find a significant genotype and age interaction in our analyses for CR cells in either area (Fig. 6E). These data suggest that AAV transduction does not significantly alter physiological programmed cell death of CR cells. Altogether, our data validate the utility of neonatal intracerebroventricular injection of Cre-dependent AAV as a tool to specifically manipulate CR cells in the adult hippocampus with minimal recombination in DG granule neurons.

Neonatal intraventricular injection of adeno-associated virus facilitates neural activity modulation of Cajal–Retzius cells in the adult hippocampus

One potential application for specific genetic manipulation in adult CR cells is to manipulate their neural activity and determine the effects on neural circuits and animal behavior. We therefore undertook a proof-of-principle study to determine whether neonatal intracerebroventricular AAV injection into the $\Delta Np73$ -Cre mice is suitable for this application. Neural activity modulation may be achieved using either optogenetics or chemogenetics. While optogenetics has a localized effect because of the limits of light delivery, chemogenetics is often used for broad or brain-wide targeting (Vlasov et al., 2018). As CR cells are widely distributed throughout the adult hippocampus, we decided to use chemogenetics to activate these cells. To this end, $\Delta Np73$ -Cre P0 pups were injected intraventricularly with 1.0×10^{12} GC/ml AAV8 carrying a Cre-dependent expression construct of hM3D(Gq)-mCherry fusion protein driven by the neuronal promoter *hSyn* [AAV8/hSyn-DIO-hM3D-mCherry (or AAV8/DIO-hM3D for short)]. At P14, ~66% of CR cells along the hippocampal fissure and ~59% of CR cells in the lower molecular layer expressed the mCherry reporter (Fig. 7A,B, Extended Data Fig. 7-1A). Robust mCherry reporter expression persisted in 7-week-old $\Delta Np73$ -Cre mice injected neonatally with AAV8/DIO-hM3D (Fig. 7C,D,

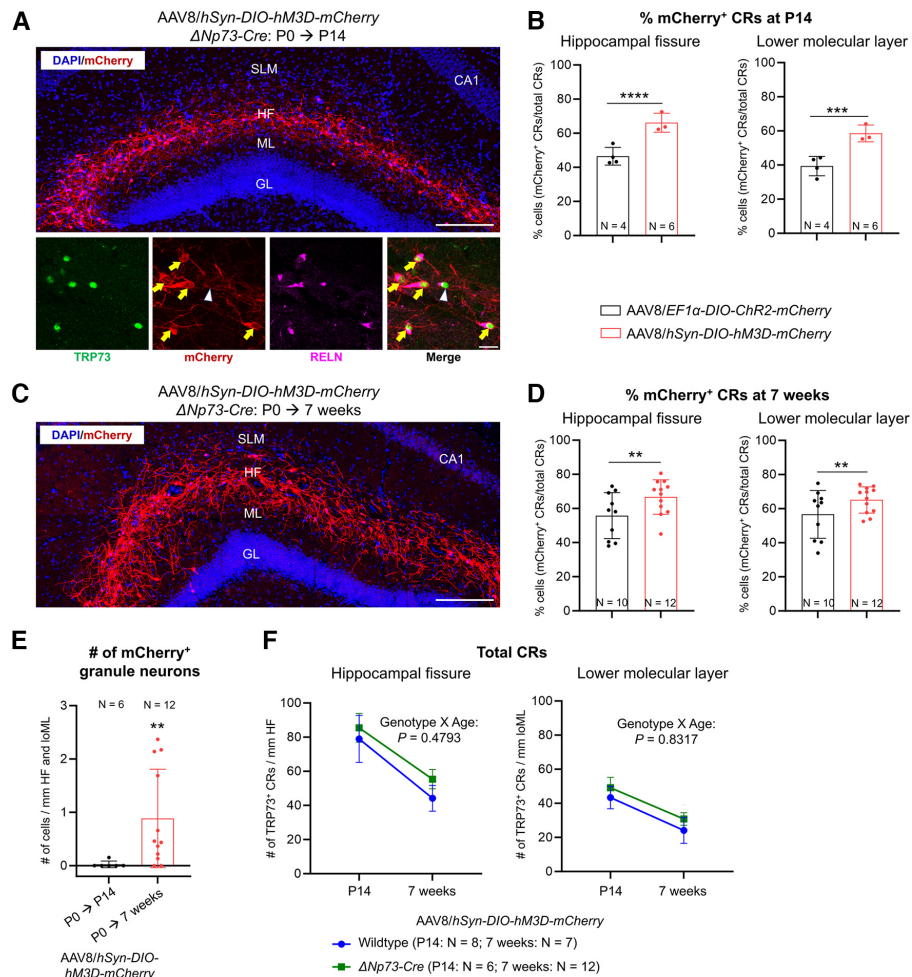


Figure 7. Neonatal intraventricular injection of AAV8/hSyn-DIO-hM3D-mCherry results in robust and highly specific transgene expression in Cajal-Retzius cells. **A**, Neonatal $\Delta Np73$ -Cre pups were injected with adeno-associated virus serotype 8 carrying a Cre-dependent expression construct for the hM3D-mCherry fusion protein under the *hSyn* promoter (AAV8/hSyn-DIO-hM3D-mCherry) and brain tissue analyzed at P14. mCherry-expressing cells also express TRP73 and RELN, confirming their identity as CR cells (yellow arrows). Some CR cells do not express mCherry as they were not transduced by the injected AAV8 (white arrowhead). Scale bars: top, 200 μ m; bottom, 20 μ m. **B**, Comparison of viral transduction efficiency in CR cells at P14 of the two different viral constructs (AAV8/EF1 α -DIO-ChR2-mCherry and AAV8/hSyn-DIO-hM3D-mCherry). **C**, Reporter expression persists for 7 weeks after neonatal injection. A representative image of a $\Delta Np73$ -Cre mouse hippocampus 7 weeks post injection with AAV8/hSyn-DIO-hM3D-mCherry is shown. Scale bar, 200 μ m. **D**, Comparison of viral transduction efficiency in CR cells at 7 weeks of age of the two different viral constructs (AAV8/EF1 α -DIO-ChR2-mCherry and AAV8/hSyn-DIO-hM3D-mCherry). **E**, Comparison of the number of mCherry⁺ granule neurons in P14 and 7-week-old $\Delta Np73$ -Cre mice neonatally injected with AAV8/hSyn-DIO-hM3D-mCherry. **F**, Quantification of the densities of TRP73⁺ CR cells in P14 and 7-week-old wild-type or $\Delta Np73$ -Cre mice neonatally injected with AAV8/hSyn-DIO-hM3D-mCherry. SLM, Stratum lacunosum-moleculare; HF, hippocampal fissure; ML, molecular layer; GL, granular layer; CA1, Cornu Ammonis 1; loML, lower molecular layer (i.e., molecular layer of the infrapyramidal blade of the dentate gyrus). Data are presented in **B**, **D**, and **E** as scatter plots with all data points shown and error bars representing $\pm$ SD; statistical analyses were performed using nested *t* tests. Each data point is an individual animal, whereby three sections were measured for each animal. Data are presented as summary data in **F** with mean $\pm$ SD, and statistical analyses were performed using two-way ANOVA with Sidak test to correct for multiple comparisons. ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. The data that demonstrate the absence of Cre-mediated recombination in the wild-type mice are shown in Extended Data Figure 7-1. Analyses of the effect of sex on AAV transduction efficiency are provided in Extended Data Figure 7-2.

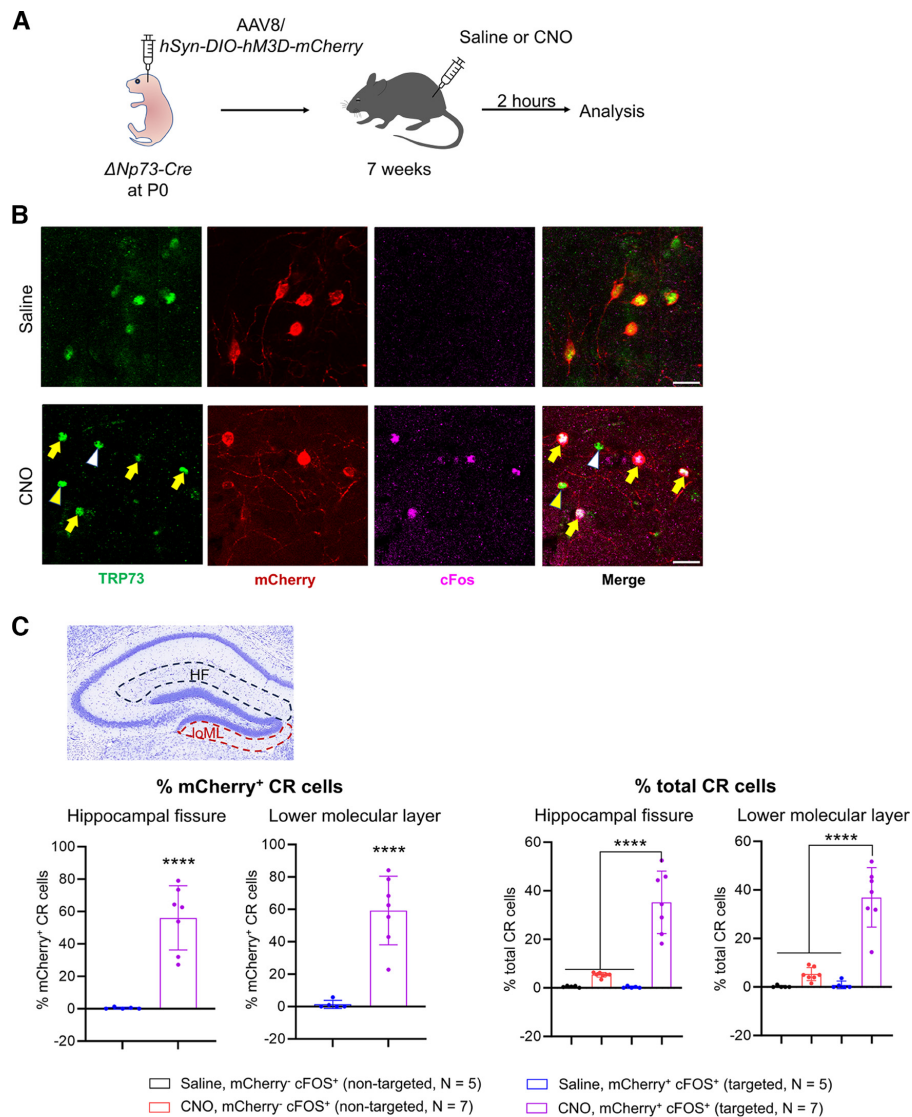


Figure 8. Chemogenetic activation of Cajal-Retzius cells in the adult hippocampus. **A**, Schematic of the experimental procedures. **B**, In 7-week-old mice neonatally injected with AAV8/*hSyn-DIO-hM3D-mCherry*, treatment of CNO results in activation of TRP73⁺ CR cells that express the chemogenetic receptor hM3D-mCherry, indicated by c-Fos immunoreactivity (yellow arrows). A CR cell with hM3D-mCherry expression but is not activated (mCherry⁺, c-Fos[−]) is indicated by the yellow arrowhead. A CR cell that does not express the hM3D receptor and is not positive for c-Fos is indicated by the white arrowhead. Scale bars, 20 μ m. **C**, Quantification of the percentage of different populations of c-Fos⁺ CR cells of mCherry⁺ or total CR cells. In each of the saline-injected control mice, 69–91 total CR cells were analyzed along the hippocampal fissure and 37–43 total CR cells were analyzed in the lower molecular layer. In each of the CNO-injected mice, 74–91 total CR cells were analyzed along the hippocampal fissure and 39–46 total CR cells were analyzed in the lower molecular layer. Data are presented as scatter plots with all data points shown and error bars representing $\pm$ SD. Each data point is an individual animal, whereby three sections were measured for each animal. Statistical analyses were performed using nested *t* test or nested one-way ANOVA with Tukey's *post hoc* test. *****p* < 0.0001. The data that demonstrate the absence of CR cell activation in the wild-type mice are shown in Extended Data Figure 8-1.

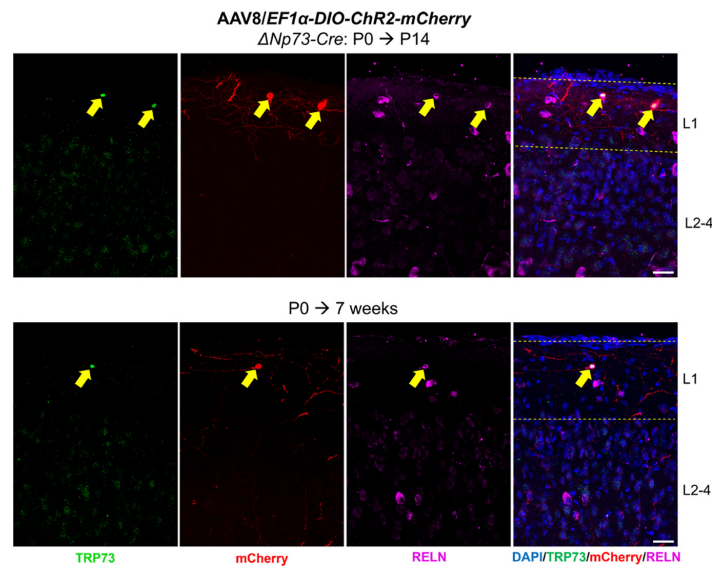


Figure 9. Effects of neonatal intraventricular adeno-associated virus injection on Δ Np73-lineage cells in the neocortex. Δ Np73-Cre pups injected at P0 with AAV8/EF1 α -DIO-ChR2-mCherry were analyzed for mCherry expression at 14 d (P14, top) or 7 weeks (bottom) post injection. Coimmunostaining of TRP73 and RELN identifies neocortical Cajal–Retzius cells that express mCherry (yellow arrows). Neocortical Cajal–Retzius cells are only found in layer 1 (L1). They are very sparse at P14 and almost completely disappear by 7 weeks of age. Scale bars, 30 μ m.

Extended Data Figs. 5-2B, 7-1B). Compared with our results using the AAV8/DIO-ChR2, the AAV8/DIO-hM3D virus transduced a significantly higher proportion of CR cells at both ages examined (Fig. 7B,D, Extended Data Fig. 7-2), suggesting that the *hSyn* promoter may be more robust than the *EF1 α* promoter for driving transgene expression in hippocampal CR cells.

At P14, only one of the six Δ Np73-Cre mice injected with AAV8/DIO-hM3D showed recombination in DG granule neurons (one cell in one of three sections examined). This became more prevalent in mice at 7 weeks of age (Fig. 7E), indicating that recombination in granule neurons still occurred using our approach. However, similar to our findings using the AAV8/DIO-ChR2, the number of recombined granule neurons in 7-week-old AAV8/DIO-hM3D-injected mice is significantly lower, while the CR cell-targeting specificity is significantly higher, than that in the Δ Np73-Cre; *LSL-tdTomato* mice of the same age (Fig. 6D), again demonstrating that neonatal intracerebroventricular AAV injection into the Δ Np73-Cre pups effectively limits recombination events in granule neurons even in adult mice. We further determined that AAV8/DIO-hM3D transduction did not significantly alter the physiological cell death dynamics of CR cells (Fig. 7F).

To test the potential application of activity modulation in CR cells, we intraperitoneally injected CNO to activate the hM3D receptors (Fig. 8A), which leads to G-protein-coupled receptor signaling, intracellular calcium release, and neuronal excitation (Armbruster et al., 2007). We quantified the percentage of CR cells with c-Fos expression as a surrogate

for neural activity (Fig. 8B). In saline-injected controls, we did not find any c-Fos expression in nontargeted (i.e., hM3D-mCherry[−]) CR cells, while only a few targeted (i.e., hM3D-mCherry⁺) CR cells exhibited c-Fos expression (Fig. 8C, Extended Data Fig. 8-1). This suggests that adult hippocampal CR cells were inactive under the specific conditions of our experiment and that CR cells expressing hM3D are not activated without CNO. Indeed, CNO administration led to a dramatic increase in the number of hM3D-mCherry⁺ CR cells with c-Fos immunoreactivity, with 55–60% hM3D-mCherry-expressing CR cells, which was ~35% of the total CR cells, being activated along the hippocampal fissure and in the lower molecular layer (Fig. 8C). Our data thus provide proof-of-principle support for neonatal intracerebroventricular AAV injection into Δ Np73-Cre mice as a strategy to manipulate CR cell activity in the adult hippocampus.

Discussion

CR cells are important organizers of cortical development in the embryonic brain, but their persistence in the adult brain, especially the hippocampus, has largely been ignored (Anstötz et al., 2018a; Causeret et al., 2021). This conceptual bias, adding to the lack of suitable technical tools to specifically manipulate adult CR cells, contributes to the paucity of information on their function in the adult brain circuits and their influence on behavior. In this study, we show surprisingly high levels of recombination events in postnatal DG granule neurons in the Δ Np73-Cre genetic crosses to reporter lines. Seeking to restrict Cre-

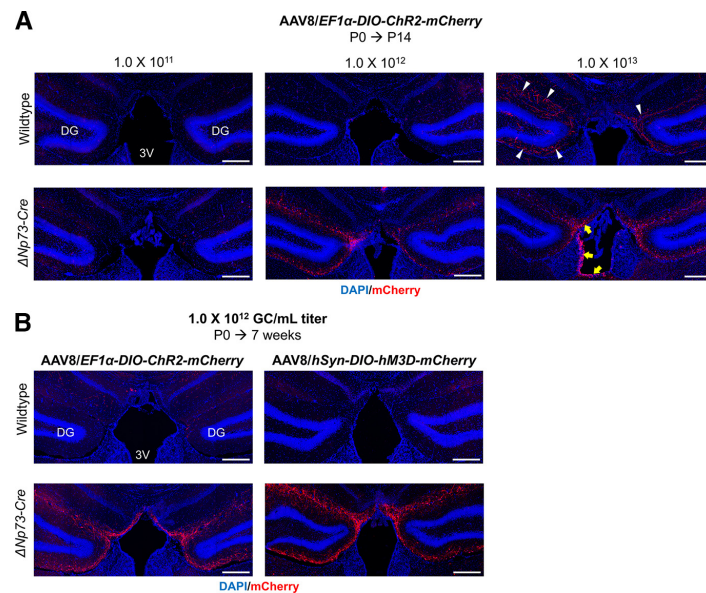


Figure 10. Effects of neonatal intraventricular adeno-associated virus injection on $\Delta Np73$ -lineage cells in the third ventricle and choroid plexus. **A**, Wild-type or $\Delta Np73$ -Cre pups injected at P0 with AAV8/EF1 α -DIO-ChR2-mCherry at different titers were analyzed for mCherry expression at P14. At 1.0×10^{13} viral titer, Cre-independent recombination was evident in the wild-type mice (white arrowheads). Additionally, at 1.0×10^{13} GC/ml viral titer, choroid plexus epithelial cells in the third ventricle (3V) of the $\Delta Np73$ -Cre mice were also transduced, evident from their mCherry expression (yellow arrows). Transduction of epithelial cells is not detectable at the 1.0×10^{12} AAV titer. Scale bars, 300 μ m. **B**, Wild-type or $\Delta Np73$ -Cre pups injected at P0 with AAV8/EF1 α -DIO-ChR2-mCherry or AAV8/hSyn-DIO-hM3D-mCherry at 1.0×10^{12} GC/ml viral titer were analyzed for mCherry expression at 7 weeks postinjection. Reporter expression is absent from ependymal and choroid plexus cells.

mediated recombination to CR cells, we develop a strategy that harnesses the efficiency of neonatal intracerebroventricular injection of viral vectors and the temporal specificity of the $\Delta Np73$ -Cre driver line. Our method is easy to implement, efficient, and versatile, offering a starting point for a wide range of experiments into CR cell function in the postnatal and adult hippocampus.

Our genetic crosses of the $\Delta Np73$ -Cre driver to reporter lines reveal unexpected recombination events in postnatal DG granule neurons. Because these recombination events vary substantially between mice and even within a single mouse, they are likely spontaneous events, rather than the $\Delta Np73$ promoter being turned on when DG granule neurons become mature. However, we cannot rule out the possibility that the $\Delta Np73$ promoter becomes active because of certain neural activity or dynamic cellular events during postnatal and/or adult hippocampal neurogenesis, resulting in Cre expression and recombination. Future studies using more animals could examine the effects of sex, housing conditions, handling, and animal behaviors on $\Delta Np73$ -Cre-mediated recombination in granule neurons. Nonetheless, our findings highlight the need for careful assessment of unwanted recombination events in genetic crosses of Cre driver lines to ensure that experimental results are generated from the intended genetic manipulation. Whether recombination outside of CR cells will confound

result interpretation should be evaluated on a case-by-case basis with consideration of the potential contribution of each cell type. While lineage-tracing studies might tolerate erroneous recombination, where additional protein markers and morphologic and/or location information are available to aid in the interpretation of results, studies involving circuit manipulation or animal behavior may be less forgiving. For example, neural activity manipulation in adult $\Delta Np73$ -Cre mice that harbor opsins or chemogenetic receptors through genetic crosses will inevitably activate both CR cells and DG granule neurons. As CR cells modulate the hippocampal microcircuits (Quattrocchio and Maccaferri, 2014; Anstötz et al., 2016, 2018b, 2022) and DG granule neurons control hippocampal information processing (Jonas and Lisman, 2014), it may be difficult to tease apart the relative contributions of the two cell types if both are manipulated simultaneously. However, with our neonatal AAV injection approach, it is now possible to selectively activate or silence adult hippocampal CRs with limited effects on granule neurons.

Our study focuses on the $\Delta Np73$ -Cre line, because of its popularity in CR cell research (Tissir et al., 2009; Ledonne et al., 2016; Riva et al., 2019; Anstötz et al., 2022; Genescu et al., 2022) and our data showing its high specificity compared with the *Wnt3a*-Cre line. However, the homozygous $\Delta Np73$ -Cre mouse model results in

knockout of $\Delta Np73$ and ablation of $\Delta Np73$ -lineage CR cells in the brain (Tissir et al., 2009). In our study, we used only the hemizygous $\Delta Np73$ -Cre mice, which do not demonstrate CR cell loss (Tissir et al., 2009) and present with similar CR cell densities in the hippocampus compared with wild-type mice. Moreover, while our study focuses only on the postnatal and adult hippocampus, we recognize that CR cells in the neocortex are also derived from the $\Delta Np73$ lineage (Ledonne et al., 2016; Riva et al., 2019; Genescu et al., 2022); therefore, our strategy will also transduce neocortical CRs. While very few neocortical CRs are present in the adult brain (Fig. 9), this is an important consideration when designing experiments, especially if the contribution of the few remaining neocortical CRs may lead to misinterpretation of results. Furthermore, choroid plexus and ependymal cells also belong to the $\Delta Np73$ lineage (Tissir et al., 2009; Marshall et al., 2016). In our hands, while the 1.0×10^{13} GC/ml viral titer did result in reporter expression within these cells, the lower 1.0×10^{12} GC/ml titer did not (Fig. 10), suggesting that careful titration of the AAV vector can reduce and even eliminate Cre-mediated recombination events in these cells.

Toward the goal of genetic manipulation of postnatal and adult CR cells, we adapted a previously developed neonatal intracerebroventricular injection method to introduce AAV vectors into the brain. Alternatively, AAVs can be introduced via stereotaxic injections. In a recent study, stereotaxic neonatal AAV injection was used to selectively ablate postnatal hippocampal CR cells in the *Pde1c*-Cre mice (Glærum et al., 2022). Compared with our fast and easy freehand neonatal injection method, stereotaxic neonatal injection is more labor intensive and time consuming, resulting in processing fewer animals per day, thereby increasing day-to-day variability. Stereotaxic neonatal injection also requires specialized surgery and injection equipment, and thus more technical training. While neonatal intracerebroventricular injection offers brain-wide transduction of the viral vector, the transduction pattern from stereotaxic injection is more localized, limiting to areas adjacent to the injection site (Kim et al., 2013). As CR cells are broadly distributed throughout the hippocampus, stereotaxic AAV injection may only transduce CR cells in a specific subregion of the hippocampus. However, such localized delivery may increase the local viral titer and allow for better control of the effective viral titer, which may explain the slightly higher transduction efficiency (~70%) in the Glærum et al. (2022) study compared with our results (~50%). However, this difference could also be because of the use of different Cre driver lines (*Pde1c*-Cre vs $\Delta Np73$ -Cre), which may have slightly different Cre expression levels and hence recombination efficiency in CR cells. Future studies applying our approach to the *Pde1c*-Cre mice or performing stereotaxic neonatal brain injections into the $\Delta Np73$ -Cre mice will provide a better comparison between the efficiencies of the two techniques and the two Cre-driver lines. In addition to neonatal AAV injection into Cre-driver mice, it may also be possible to genetically manipulate CR cells in the adult brain using the inducible Cre/ERT2 system, whereby Cre is activated after tamoxifen treatment (Metzger et al., 1995; Schwenk et al., 1998). The

Fzd10-Cre/ERT2 line shows inducible Cre-mediated recombination in CR cells during embryonic development (Metzger et al., 1995; Schwenk et al., 1998). However, its specificity for CR cells in the postnatal and adult brain has not been characterized, and therefore, its utility remains to be tested.

A limitation of our approach is that we only achieve Cre-mediated recombination in ~50% of postnatal CR cells. This may limit the usefulness of our approach in conditional gene knock-out studies, both because of its relatively low efficiency and because the volume of the region transduced by the AAV may vary. Whether a ~50% recombination rate is sufficient for gene overexpression, lineage tracing, circuit manipulation, and behavioral analysis should be evaluated on a case-by-case basis. In our hands, increasing the viral titer by 10-fold significantly increased viral transduction efficiency but led to increased animal morbidity and spontaneous recombination of the viral construct in DG granule neurons. Further optimization may be needed by using different Cre-driver lines and/or other AAV serotypes. Future studies may adapt our strategy to study the role of CR cells in the adult hippocampus via optogenetic and chemogenetic approaches. Previous studies have only stimulated CR cells optogenetically in ex vivo tissue (Quattrocchio and Maccaferri, 2014; Riva et al., 2019; Anstötz et al., 2022), or chemogenetically in vivo at early postnatal age from P1–P3 (Genescu et al., 2022). As a result, we know very little about the functions of CR cells in the adult brain circuit and animal behavior. Of note, we found that few CR cells in the adult hippocampus express c-Fos under basal condition, suggesting that they are inactive under the specific conditions of our experiment. Because of this low baseline activity, activating even a small number of CR cells using our approach may impact the hippocampal circuit and animal behavior. While optogenetic and chemogenetic manipulation of adult CR cells followed by behavioral studies is beyond the scope of our study, our framework offers a starting point for others to recognize the persistence of CR cells in the adult hippocampus and begin to address their functions.

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