

**A redox chemistry-based function for Parkinson disease-linked parkin
confers direct, anti-oxidant activities in mammalian brain**

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Abstract

Early-onset Parkinson disease, of which the best studied and most common cause are bi-allelic mutations in the *PRKN* gene, is characterized by an age of onset before 40 years. Parkin-deficient patients show slow progression, excellent responsiveness to L-dopa therapy, and are generally spared cognitive decline. At autopsy, *PRKN*-linked Parkinson disease is further distinguished by relative selectivity in cell loss, namely of dopamine producing neurons in the human brainstem, and the general absence of Lewy body inclusions. Since its discovery two decades ago, the field has focused on the function of parkin as an E3 ubiquitin ligase and its related role in mitophagy. However, its essential, neuroprotective function in ageing human midbrain and the mechanisms by which wild-type parkin preserves dopamine cell health in aged humans are yet to be elucidated. I hypothesized that parkin confers neuroprotection due to a redox function by its many cysteines (7.5%). We first focused on parkin's biochemistry, reporting a shift from solubility to a nearly insoluble, aggregated state in adult control brain after age 40 years. We detected cysteine-based, post-translational modifications of parkin in response to oxidative stress, and characterized a novel, redox chemistry-based function: Through its own oxidation parkin reduced hydrogen peroxide *in vitro*. I validated this finding by showing its elevation in parkin-deficient, human brain. Wild-type parkin also participated in dopamine metabolism through the conjugation of reactive radicals at several of its cysteines, which augmented the generation of melanin. (Chapter 2). In addition, we demonstrated parkin's heretofore unknown, antioxidant function in the cytosol using cellular paradigms and select genetic as well as toxin-based mouse models that featured elevated oxidative stress. Moreover, we uncovered parkin's contribution to the wider

thiol network, namely through an apparent feedback loop with glutathione metabolism (Chapter 3). Lastly, I developed and investigated a bi-genic (*prkn*^{-/-}/*Sod2*^{+/-}) model in an attempt to restage the pathogenesis of early-onset parkinsonism in mice; there, we detected a rise in systemic, oxidative stress and in total nitrotyrosination profiles of the brain, but did not observe any dopamine neuron loss in the midbrain. In accordance, the behavioural characterization of these animals did not reveal any motor abnormalities (Chapter 4). Based on this previously unknown function for parkin in redox biology, I envision three future research directions: a) additional studies to delineate the full range of oxidative modifications of parkin *versus* neutralization of radicals; this, to better define the distinct pathogenesis of parkin-linked Parkinson's when compared to other forms of the disorder; b) structural studies of its oxidative modifications *in vitro* and renewed attempts of staging parkin-linked dopamine cell death *in vivo*; and c) and importantly, the exploration of cause-directed therapy based on parkin's redox functions to preserve dopamine neurons of the human midbrain throughout adulthood.

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“Any man could, if he were so inclined, be the sculptor of his own brain” Santiago Ramón y Cajal, Advice For a Young Investigator, 1897

List of Manuscripts:

1. **Oxidative Modifications of Parkin Underlie its Selective Neuroprotection in Adult Human Brain.....82**
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3. **Validation of Anti-Oxidant Function by Parkin in Mammalian Brain Through the Characterization of a Bi-genic *prkn*^{-/-}//*Sod2*^{+/-} Mouse Model. (In preparation).....197**

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List of Abbreviations:

6-OHDA	6-hydroxydopamine
aa	Amino acid
AAV	Adeno-associated virus
Aco2	Aconitase-2
AD	Alzheimer's disease
AIMP2	Aminoacyl tRNA synthetase-complex interacting multifunctional protein2
FBP1	Far upstream element-binding protein 1
ARJP	Autosomal recessive juvenile parkinsonism
ARPD	Autosomal recessive PD
AT	Aminotriazole
ATP	Adenosine triphosphate
A β (1-42)	Amyloid beta peptide 1-42
BA	β -actin
BAC	Bacterial artificial chromosome
BBB	Blood brain barrier
Bp	Base pair
BSO	Buthionine sulfoximine
C	Cysteine residue
CaMKII	Calcium/calmodulin-dependent protein kinase II
CBA	Chicken β -actin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine

CD	Circular dichroism
CHO	Chinese hamster ovary
cM	Centimorgan
CMV	Cytomegalovirus
CMVE	Cytomegalovirus enhanced
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
COR	C-terminal of ROC
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Cybrid	Cytoplasmic hybrid
DA	Dopamine
DAT	Dopamine transporter
DATATOP	Deprenyl And Tocopherol Antioxidative Therapy of Parkinsonism
DBS	Deep brain stimulation
ddc	Dopa decarboxylase
DLS	Dynamic-light-scattering
DTT	Dithiotreitol
DUB	Deubiquitinating enzymes
EDTA	Ethylenediaminetetraacetic acid
elav	Embryonic lethal – abnormal vision
EOPD	Early-onset parkinsonism
GABA	Gamma-Aminobutyric acid

GBA	Glucocerebrosidase
GCLC	Glutamate-cysteine-ligase-catalytic
GCLM	Glutamate-cysteine-ligase-modifier
GI	Gastrointestinal
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx1/2	Glutaredoxin 1/2
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H ₂ O ₂	Hydrogen peroxide
HECT	Homologous to the E6-AP carboxyl terminus
HEK293	Human embryonic kidney
HHARI	Human homolog of Ariadne
hLRRK2	HumanLRRK2
HMW	Higher molecular weight
HOIP	Heme-oxidized IRP2 ubiquitin ligase-1 (HOIL1)-interacting protein
HRP	Horseradish peroxidase
I	Isoleucine residue
IAA	Iodoacetamide
IBR	In-between-RING
IFI	Immunofluorescence
IHC	Immunohistochemistry
IP	Immunoprecipitation

iPSCs	Induced pluripotent stem cells
IV	Intravenous
K	Lysine residue
kgF	Kilogram-force
L-DOPA	Dopamine precursor
LAMP3	Lysosome-associated protein-3
LB	Lewy body
LC	<i>Locus coeruleus</i>
LC-MS/MS	Liquid chromatography-based mass spectrometry
LGP	Lateral globus pallidus
LN	Lewy neurite
LRR	Leucine Rich Repeat
LRRK2	Leucine rich repeat kinase 2
LUBAC	Linear ubiquitination chain assembly complex
Maneb	2-(dithiocarboxy)aminoethylcarbamodithioato(2-)-kS, kS' manganese
MAO	Monoamine oxidase
MCP-1	Monocyte chemotactic protein-1
MDV	Mitochondrial derived vesicle
MFN1/2	Mitofusuin 1/2
MGP	Medial globus pallidus
Miro	Mitochondrial Rho GTPase
MitAP	Mitochondrial antigen presentation
mLRRK2	Murine LRRK2

MnSOD	Mitochondrial superoxide dismutase-2
Mono	Monoubiquitination
MPP	Mitochondrial processing peptidase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
M_r	Molecular weight
MRI	Magnetic resonance imaging
MSA	Multiple system atrophy
mtCK	Creatine kinase
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting signal
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NAT	Noradrenergic transporter
NBT	Nitro-blue-tetrazolium
ND	No data
NEM	N-ethylmaleimide
NF- κ B	Nuclear factor κ B
NLRP3	Nod-like receptor family, pyrin domain-containing 3
NM	Neuromelanin
NN	Blood mononuclear cells
NO	Nitric oxide
NOX2	NADPH oxidase
nse	Neuron specific enolase
O_2^-	Superoxide radical

OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PACRG	Parkin co-regulated gene
Paraquat	N, N'-dimethyl-4,4'-bipyridiniumdichloride
PARIS	Parkin interacting substrate
PARL	Presenilins-associated rhomboid-like protein
PD	Parkinson disease
PDD	PD-related dementia
PDGF- β	Platelet derived growth factor- β
PGK	Phosphoglycerate kinase
PINK-1	Phosphate and tensin homolog (PTEN)-induced serine/threonine kinase 1
Pitx3	Paired-like homeodomain3
PMI	<i>Post mortem</i> interval
POLG	Exonuclease domain II of DNA polymerase subunit gamma
Poly	Polyubiquitin chain addition
PRKN	Parkin
Prp	Prion protein promoter
PTM	Post-translation modification
PY	Cortical pyramidal neurons
r-	Recombinant
r-parkin _{321C}	C-terminal RING2 peptide
RBR	RING-between-RING
REM	Rapid-eye-movement

REP	Repressor element of parkin
RES	Reactive electrophilic species
RING	Really Interesting New Gene
RN	<i>Raphe nuclei</i>
RNAI	RNA interference
RNS	Reactive nitrogen species
ROC	Ras of Complex
ROS	Reactive oxygen species
ROSA26	ROSA β geo26P locus
SAG	Sensitive-to-apoptosis gene
Slca6a3	Solute carrier family 6a3;
SNC	<i>Substantia nigra pars compacta</i>
SNCA	α -synuclein
SNDA	<i>Substantia nigra</i> DA neurons
SNr	<i>Substantia nigra pars reticulata</i>
Sod2	Superoxide-oxide dismutase
ST	<i>Salmonella typhimurium</i>
STING	Stimulator of interferon genes
STN	Subthalamic nucleus
SYN 1	Synapsin 1
TFAM	Transcription factor A, mitochondrial
TH	Tyrosine hydroxylase
Thy-1	Thy-1 cell surface antigen

TLR2	Toll-like receptor 2
TOM20/40	Translocase of outer mitochondrial membrane 20/40
Trx1	Thioredoxin 1
UBA	Ubiquitin associated
Ubl	ubiquitin-like domain
UIM	Ubiquitin interacting motifs
UPD	Unique parkin domain (RING0)
UPDRS	Unified Parkinson's Disease Rating Scale
UPS	Ubiquitin-proteasome system
USP	Ubiquitin specific peptidase
UTR	Untranslated region
VMAT2	Vesicular monoamine transporter 2
VPS35	Vacuolar protein sorting-associated protein 35
VTA	Ventral tegmental area
WT	Wild-type

Chapter 1: General Introduction

1.1 Overview of Parkinson disease

Parkinson disease (PD), first described by Dr. James Parkinson in “An Essay on the Shaking Palsy” (*Paralysis Agitans*) in 1817¹ and later renamed ‘*la maladie de Parkinson*’ in 1890 by Dr. Jean-Martin Charcot, is a complex neurodegenerative disorder that manifests with several motor symptoms; it also includes non-motor features and frequently leads to cognitive impairment. Parkinson disease, is the second commonest neurodegenerative disease following Alzheimer’s disease (AD) and the second most frequently diagnosed movement disorder after essential tremor².

Motor features in parkinsonism are the result of reduced dopamine transmission within the basal ganglia; in PD, this is caused by the progressive loss of dopamine neurons in the *Substantia nigra pars compacta* (SNc)³. A person is diagnosed with probable PD once two of three motor symptoms are present (the classical triad encompasses tremor, bradykinesia and rigidity), L-dopa-responsiveness has been established, and progression of illness is documented⁴. Given the heterogeneity of the disease and its mixed clinical phenotype, a definitive diagnosis is usually established by *post mortem* autopsy; however, using the Unified Parkinson’s Disease Rating Scale (MDS-UPDRS), neurologists can assess clinical staging of both motor and non-motor features in order to provide patients with a working diagnosis⁵. There are situations in which PD is more difficult to diagnose based on symptoms alone. This is more likely to happen in the early stages of the disease process. In these cases, it is possible to use dopamine (DA) transporter-based single-photon emission computer tomography⁶ to visualize the loss of DA terminals at the striatum. Nevertheless, this approach does have limitations, for

example in that it cannot differentiate between PD and other diseases that lead to progressive parkinsonism, but it can separate it from various forms of tremor.

The histopathological substrates of typical PD are a reduction in the number of dopaminergic neurons in the SNc and the formation of intracytoplasmic and axonal inclusions within at risk as well as surviving neurons. Magnetic resonance imaging (MRI) and other imaging techniques, on the other hand, can be used to differentiate PD from other conditions, such as those caused by structural lesions. The condition is frequently classified as a progressive, extrapyramidal disorder without readily apparent etiology (“idiopathic”). However, parkinsonism is a heterogeneous clinical syndrome. Other extrapyramidal disorders that are associated with parkinsonism have different etiologies, such as trauma, drug exposure (both illicit and prescribed), viral infection, vascular abnormalities, and others⁷; these are referred to as ‘secondary parkinsonism’. Fewer than 10% of clinically diagnosed parkinsonian cases are considered secondary in nature, less than 10% are truly ‘monogenic’, with the remainder being classified as ‘idiopathic’⁸.

Ageing is thought to be the number one risk factor for developing PD⁹. Most PD cases occur after the age of 55 years, affecting 1% of the population over 60, approximately 3% after age 70, and 5% of individuals over 85 years¹⁰. The prevalence of the disease is highest in countries with a population shift toward the elderly, affecting males more than females¹¹. There are three categories of parkinsonism in regards to the age at which the disease begins: Juvenile-onset PD (<20 years); early-onset, adult PD (20-40 years); and

typical, late-onset adult PD (age >55 years) with the period between ages 40 and 55 falling into a grey zone. It is important to note, and as it relates to the topic of this thesis (parkin), cases of early onset-autosomal recessive parkinsonism (EOPD), which clinically look like typical PD, occur infrequently, most commonly due to mutations in genes such as *PRKN*, *PINK1* and *DJ-1*. Unlike sporadic or idiopathic PD cases, these patients develop the disease not only with an earlier age of onset, but they also typically present with motor abnormalities that include dystonia and seem to be spared cognitive decline. Additionally, in parkin-linked cases the disease process appears localized to dopamine-producing neurons^{12,13}. These cases will be discussed in further details in later sections.

The unclear cause of PD pathogenesis and the growing evidence for interplay between environmental factors and multiple genes contribute to the difficulty in finding a cure. The chronically progressive nature of PD renders affected patients ultimately less mobile -despite best medical and surgical therapy- and prone to developing pneumonias, cancer (e.g., melanoma) and cardiovascular disease¹⁴. To date, currently available treatments for PD are designed to help manage motor symptoms, as first introduced in the early 1960s¹⁵⁻¹⁷, in order to make disease progression more manageable. Importantly, current therapies do not prevent, slow or stop disease progression.

1.2 Etiology

PD cases can be categorized as principally secondary (<10%), mostly sporadic or idiopathic (80%), and genetic, *i.e.*, familial (<10%); however, evidence points to a

combination of genetic susceptibilities and environmental factors as contributors to the pathogenesis of the disease in all three categories^{18,19}.

1.2.1 Sporadic Parkinson disease

Environmental factors have been implicated in the pathogenesis of typical, sporadic PD. Pesticide exposure (for example rotenone and paraquat), exposure to heavy metals (for example manganese), concussions from repeated head injuries (for example in heavy contact sports such as boxing), and neuroleptic drug exposure, are a few examples^{8,20}. A better understanding of how environmental factors and PD-linked genes interact could shed more light on the etiology of PD, as most studies so far are correlative making it difficult to establish a direct causal link to PD.

It is noteworthy that certain environmental and lifestyle factors have been demonstrated to correlate with a negative risk of developing PD, for example caffeine consumption and smoking^{21,22}, which have been hypothesized -among other mechanisms- to affect gastrointestinal (GI) functions.

1.2.2 Familial Parkinson disease

In 1997, the first PD gene was identified encoding α -synuclein (*SNCA*)²³. Since then, multiple genes have been mapped, and linked to either autosomal dominant or autosomal recessive forms of PD. Studies have not only identified PD-linked and monogenetically ‘causative’ alleles, such as for *SNCA*, *LRRK2*, *PRKN*, *PINK1* and *DJ-1*, but have also

generated a number of genetic variants (coding and non-coding) that confer increased susceptibility to the disease^{24,25}. For example, in recent years, mutations in the glucocerebrosidase (*GBA*) gene, which has been implicated in Gaucher's disease (homozygous mutations)²⁶ has also been identified as causing an increased risk of developing PD. Research focusing on these genes, their cellular function and the pathways they mediate is of crucial importance to the development of cause-directed therapies.

The following graph⁸ summarizes the heterogeneity of PD and the interplay between genetic and environmental factors (**Figure 1.1**).

Genetic factors

Environmental factors

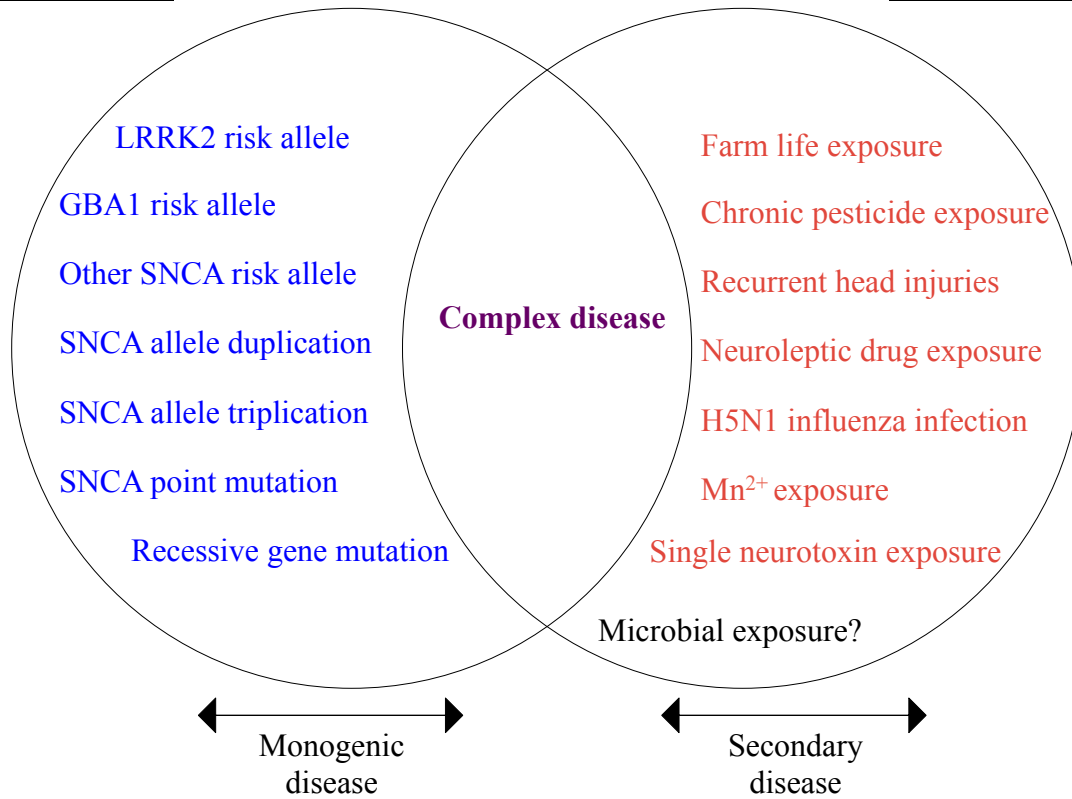


Figure 1.1: The genetic and environmental interplay in PD pathogenesis highlights its heterogeneity.

A Venn-diagram displaying known etiologies underlying clinical diagnosis of PD. To the right are environmental factors encountered by affected individuals at any given age leading to secondary parkinsonism. To the left are rare monogenic variants of PD that lead to familial cases, genes discussed in section 1.8. The intersection between the genetic and environmental factors, highlights the complexity and heterogeneity of the disease. Modified from Kitada et al., 2012.

1.3 Clinical manifestations of disease

PD is generally viewed as a disorder characterized by motor deficits; however, several non-motor aspects have been identified, accompanying or preceding the motor symptoms. These manifestations will vary depending on the patient's sex, age, stage of disease and comorbidities.

1.3.1 Motor symptoms

Parkinson disease manifests with several motor symptoms, including muscle rigidity, resting tremor, postural instability, and slowed movement. The onset of the condition is commonly unilateral and often includes other types of abnormal movements such as limb dystonia, and postural tremor. The progression of PD takes place in several stages and some symptoms are more likely to appear in the later phase of the condition, including bilateral manifestations, levodopa-induced dyskinesia, and even psychosis and dementia. Some of these are considered side effects of treatments. A diagnosis is given based on the existence of bradykinesia and resting tremor and/or rigidity signs. Observable responses to therapy based on dopaminergic neurotransmission are supportive features of the correct diagnosis²⁷. The motor deficits are linked to disruption in basal ganglia circuitry within the direct and indirect pathways due to neuronal loss in the SNc.

1.3.2 Non-motor symptoms

PD also causes non-motor symptoms such as depression, affecting up to 70% of all PD subjects before onset of their illness, or thereafter²⁸, anxiety, insomnia, constipation and other impairments in GI function, fatigue, hyposmia, rapid-eye-movement sleep (REM)

behaviour disorder (possibly due to dysfunction of *locus coeruleus* (LC) circuitry²⁹), dysarthria, dysautonomia of genitourinary functions, obsessive compulsive disorders (from excess dopamine stimulation during therapy), and dementia, among others^{2,27,30}. PD patients are believed to be six times more likely to develop dementia when compared to the general population³¹. It is worth noting that non-motor features may appear prior to motor deficits by up to 10 years, and can be considered prodromal symptoms of PD, adding to the complexity of the disease and affecting its severity³².

1.3.3 Braak and Del Tredici hypothesis of disease manifestation

Two of the prodromal features of PD that have gained strong ground and interest in recent years are hyposmia and gastrointestinal impairments. It is hypothesized that infections, of bacterial or viral origin, can enter the host through the nose or the gut, two sites of possible interactions with the environment, targeting dopaminergic neurons and resulting in the pathogenesis of PD. This “dual hit” hypothesis was established by Braak and Del Tredici suggesting that the disease process originates caudally (GI tract) and rostrally (olfactory system) and, as it progresses, reaches motor-related areas of the brain, such as the SNc (through the vagus nerve connected to the dorsal motor nucleus CNX in the medulla)^{33,34}. This was informed by the observed presence and distribution of disease-related Lewy pathology in 41 subjects examined *post mortem* (see below). This staging of PD is reinforced by the fact that early-on in the disease >75% of the patients experience hyposmia and >50% report constipation or other symptoms of GI impairment^{35,36}.

1.4 Neuropathology of PD

The diagnosis of PD from a neuropathological perspective involves detecting substantial (usually >60 per cent) dopaminergic neuronal loss in the SNc³⁷, along with the presence of Lewy bodies (LB), and eosinophilic inclusions. It should be noted that the reason for which Lewy bodies appear in PD is not currently fully understood. On the other hand, it was shown that misfolded α -synuclein is a key component of the radiating filaments and can also be found as Lewy neurites (LN), which has led to the classification of some forms of PD as a synucleinopathies³⁸.

Even though it seems like the nigrostriatal pathway is predominantly affected clinically in PD, other areas and nuclei are also impacted, which help explain the complexity of the disease and its clinical manifestations.

1.4.1 The nigrostriatal circuitry

The motor features of PD are caused by neuronal loss in the SNc, which results in a substantial loss of striatal dopamine content (nigro-striatal pathway). The loss of this subset of neurons, and the subsequent decreased in available dopamine, is observed as a decrease in neuromelanin and the black staining that accompanies its presence in dopaminergic cells³⁹. The nigro-striatal pathways is a major component of the basal ganglia circuitry, which when compromised affects voluntary movement initiation⁴⁰. Through direct and indirect pathways, mediated via different subtypes of dopamine receptors (D1 and D2 respectively), dopamine-producing neurons of the SNc project to the caudate and putamen nuclei of the striatum, which ultimately signals motor

commands to the thalamus. The signal is then transferred to the motor cortex and down the brainstem to the body. In PD, the degeneration of SNc neurons results in loss of striatal projections and dopamine availability, resulting in a dampened thalamic signal and decreased motor output^{41,42}. It is important to note that motor deficits appear in patients when approximately >60% of the SNc dopamine neurons are lost^{37,43-45}.

The following graphical depiction summarizes the changes in the basal ganglia functional organization in PD⁴¹ (**Figure 1.2**).

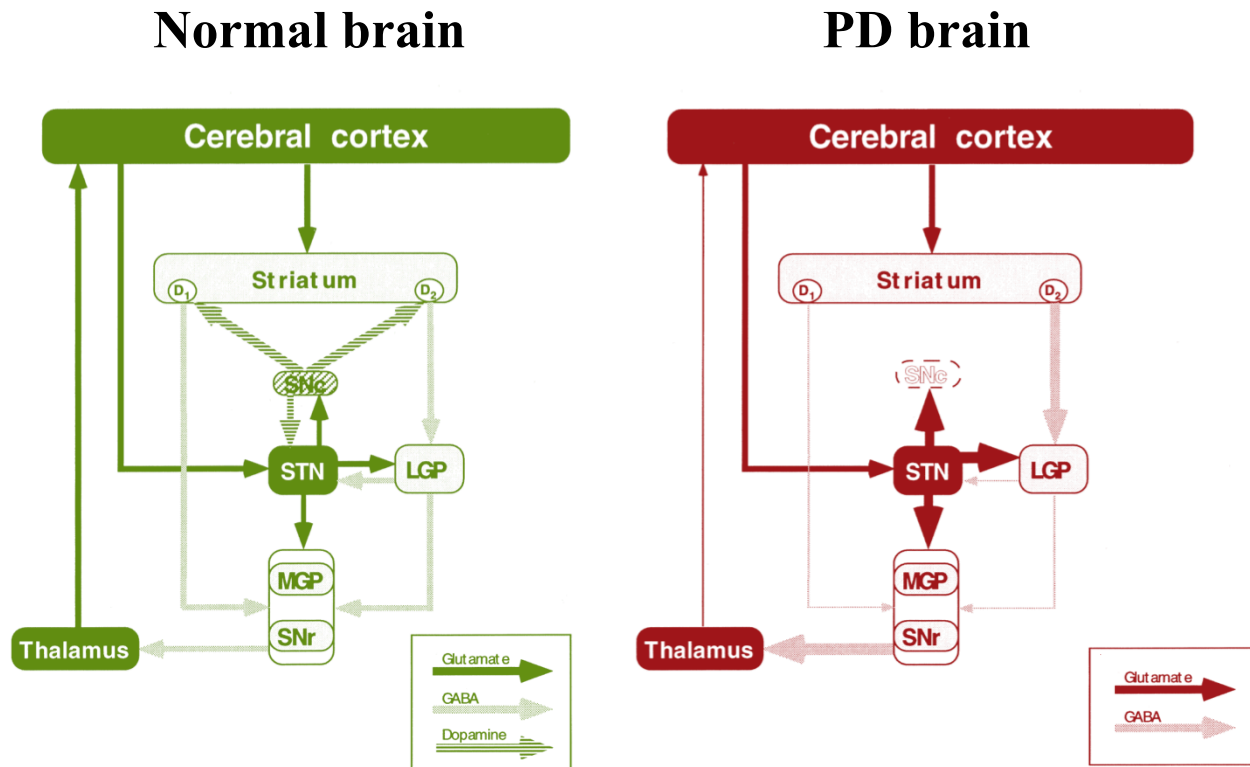


Figure 1.2: Changes in basal ganglia functional organization in PD.

In PD, the loss of SNc projections to the striatum, and the decrease in dopamine availability, induce changes to the basal ganglia circuitry, that underlie the motor symptoms. The dampened thalamic response to the motor cortex observed in PD brains results from one, the decrease in striatal signaling; and two, the increase in GABAergic outputs from the MGP and SNr, due to STN hyperactivity. The STN hyperactivity can also be explained by the decrease of SNc projections to the nucleus, the decrease in inhibitory effects on the nucleus by the LGP, and other direct projections to the nucleus such as the glutamatergic cortico-subthalamic pathway. Relative thickness of arrows indicate the degree of activation. SNc, substantia nigra pars compacta; STN, subthalamic nucleus, LGP, lateral globus pallidus; MGP, medial globus pallidus; and SNr, substantia nigra pars reticulata. Modified from Blandini et al., 2000.

1.4.2 Other affected areas

Several other nuclei or neuronal clusters exhibit diminished neuronal loss when compared to the SNc. The ventral tegmental area (VTA) comprised of dopaminergic cluster of neurons making up the mesocortical and mesolimbic pathway, are also affected, to a lower extent, in PD⁴⁶. Additionally, the LC, containing noradrenergic neurons implicated in physiological response to stress or startle, and the *raphe nuclei* (RN), containing serotonergic neurons and part of the reticular formation, may be compromised in PD patients. Likely, the cellular loss in these areas is implicated in the non-motor features of PD⁴⁷. It is worthy to note that in parkin-linked parkinsonism, the disease process leads to loss of neurons mostly in the SNc and LC nuclei¹².

1.4.3 Susceptibility of SNc dopaminergic neurons

Why specifically the SNc dopaminergic neurons die remains incompletely understood. However, several known features of the SNc dopaminergic neurons make them more vulnerable to degeneration, when compared to their neighbouring VTA dopamine-producing neurons.

One, they control their pacemaking activity through L-type voltage-gated calcium channels, as opposed to the voltage-gated sodium channels of the VTA. Calcium plays an important role in the function of mammalian cells and calcium dysfunctions are associated with PD. For instance, in normal synaptic activity (outside of DA neurons), the concentration of calcium at the intracellular level increases gradually and has no effects on neuronal health. On the other hand, DA neurons can create high levels of intracellular

calcium during pacemaking activity, resulting in mitochondrial dysfunction and oxidative stress, negatively impacting adenosine triphosphate (ATP) production and cell viability^{48,49}. The findings presented above suggest that a continuous calcium overload in DA neurons may lead to their selective vulnerability. This is supported by the fact that the dopamine neurons in the VTA, which do not depend on calcium channels, are for the most part preserved⁵⁰.

Two, the neuroanatomical structure and bioenergetic demands of the SNc dopaminergic neurons heighten their vulnerability to degeneration. This subset of neurons display elevated bioenergetics demands. Their extensive arborization lends them to exhibit higher number of mitochondria across their axons. As a result, they present with higher basal rate of mitochondrial oxidative phosphorylation (OXPHOS), lower reserve capacity and elevated basal oxidative stress⁵¹. Work by the Trudeau lab shows that manipulating the SNc dopamine neurons' arborization, by acting on axon guidance pathways with Semaphorin 7A, reduced their elevated OXPHOS and vulnerability to cell death post neurotoxin treatments with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone⁵¹, two toxins that affect electron transport chain function. Furthermore, structurally the SNc DA neurons rely on the LC noradrenergic neurons for trophic support. Therefore, it is suggested that the evidence of LC neurons loss in PD patients may explain the increased vulnerability of SNc DA neurons⁵².

Three, in addition to biophysical properties described above, these neurons must also handle dopamine metabolism. DA metabolism in long-lived primates, such as humans,

occurring within these neurons induces several pathways of stress, suggesting that DA itself is a source of oxidative stress, and hypothesizing that DA metabolism associated stress could contribute to further vulnerability. Following its synthesis from tyrosine by tyrosine hydroxylase (TH), DA is stored in synaptic vesicles. However, excess cytosolic DA is readily metabolized, by monoamine oxidase (MAO) or auto-oxidizes. This process generates large amount of reactive oxygen species (ROS)³⁹. Further DA oxidation steps generate the rather unstable electron-deficient dopamine 0-quinone, aminochrome and 5,6-indolequinone. These potentially harmful species contribute to the neurodegenerative processes by inducing mitochondrial dysfunction, protein aggregation, dysfunction in protein degradation pathways and oxidative stress (pathways that will be briefly discussed in the next section)⁵³. These oxidative processes alter mitochondrial function and brain mitochondria in particular⁵⁴; they can modify several PD-related proteins, including parkin, DJ-1, α -synuclein, and superoxide-oxide dismutase (Sod2)⁵⁵. Furthermore, aminochrome can be redox-cycled that generates superoxide radical (O_2^-) and depletes cellular nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), ultimately generating neuromelanin deposits in human SNc⁵⁶. Neuromelanin has been suggested to offer a neuroprotective effect by sequestering harmful redox metals, toxins and excess cytosolic catecholamines away from the cell. Nevertheless, it is also hypothesized that the breakdown of neuromelanin in degenerating neurons, release its content and contribute to neuroinflammation⁵⁷.

1.5 Pathophysiological processes of PD

In recent years, advancement in research has solidified evidence pointing to the role of protein aggregation, neuroinflammation, mitochondrial dysfunction, oxidative stress, and dopamine metabolism (discussed above) in the pathogenesis of PD.

1.5.1 Proteotoxic stress: aggregation and mishandling

Aberrant accumulation and processing of damaged proteins is a pathogenic pathway shared by many neurodegenerative diseases, including PD. This process starts with protein aggregation, seen as the tendency of some native proteins to become increasingly insoluble, changing in conformation, polymerizing and resulting in intracellular aggregates. These are known to be associated with neuronal dysfunction and loss. What remains a major unanswered question in the field is whether these proteinaceous inclusions are protective, incidental or pathogenic. The protein aggregation is also accompanied by a cellular response. As a result of misfolded proteins' accumulation, the cell's ubiquitin-proteasome system (UPS) is activated. The misfolded proteins are degraded in an ATP-dependent manner through ubiquitination. Hence, a dysfunctional UPS would add to the accumulation of cytotoxic protein leading to increased cell vulnerability to degeneration⁵⁸. In addition, the identification of 20S proteasome activity deficits in idiopathic PD patients' brains has been reported⁵⁹.

A feature to PD is the propensity of α -synuclein to aggregate, forming oligomers and progressing to insoluble fibrils, the major constituents of Lewy bodies⁶⁰. Several factors contribute to the predisposition of α -synuclein to aggregation, including post-translational

modifications, such as C-terminal truncation, phosphorylation and nitration⁶¹, mutations⁶², as well as interaction with dopamine, which stabilizes the fibrils forming dopamine- α -synuclein adducts⁶³.

1.5.2 Neuroinflammation

With neuronal loss and cell death comes chronic neuroinflammation. This process is controlled by microglia, the immune cells of the brain, as well as astrocytes and oligodendrocytes⁶⁴. Both sporadic and familial PD patients have shown increased SNc-specific microglia activation⁶⁵. Similar results were observed in PD animal models, for example mice treated with MPTP⁶⁶, and mice treated with 6-hydroxydopamine (6-OHDA)⁶⁷. Additionally, over-activation of microglia can result in the release of ROS, which adds to the neurotoxicity; coupled to the activation of NADPH oxidase (NOX2), which amplifies ROS production even further and initiate redox signalling leading to an increase in pro-inflammatory responses. Neuromelanin, discussed in section 1.4.3, can also induce SNc microglia activation upon its release from dying neurons, further adding stress to the already vulnerable DA neurons.

Furthermore, several PD-linked proteins have been implicated in neuroinflammatory processes that mediate PD pathogenesis. Published work, including from our team, points to a role for LRRK2 in modulating the brain's immune response^{68,69}. LRRK2 increases pro-inflammatory cytokine release from activated microglia⁷⁰, and its inhibition attenuates microglial inflammatory processes⁷¹. Alternatively, extracellular α -synuclein can act as an agonist to Toll-like receptor-2 (TLR2), which activates microglia

inflammatory pathways⁷², and an increase in activated microglia is observed in both the striatum and SNc of WT human α -synuclein overexpressing mice⁷³. When it comes to autosomal recessive PD genes, DJ-1 expression is unregulated in reactive astrocytes in PD patients⁷⁴; and parkin deficiency affects glial function and increases vulnerability to inflammation-related nigral degeneration^{75,76}.

1.5.3 Mitochondrial dysfunction and oxidative stress

The idea that mitochondrial dysfunction and oxidative stress are implicated in the pathogenesis of PD came from the observation that MPTP, rotenone and paraquat, three mitochondrial complex I inhibitors, generating high levels of ROS, were able to recapitulate parkinsonian phenotypes in animal models^{77,78}. Mitochondria are organelles that are highly abundant in high-energy demanding tissues such as hearts, skeletal muscles and brains. The main role of mitochondria is energy metabolism. While mitochondria generate ATP during OXPHOS, which plays a major role in several cellular processes, they are also the key cellular source of ROS production. At low concentration ROS are implicated in cellular signalling pathways; however, if they reach uncontrolled high levels they can participate in the regulation of cell destructive pathways and calcium cytotoxicity. Therefore, abnormal mitochondrial performance may influence selective DA neurons degeneration⁷⁹.

Special consideration must be given to complex I, which has an important role in the respiratory process of the cell, and a deficiency in the complex can lead to a substantial reduction in energetic functions, which in turn leads to mitochondrial instability. Furthermore, much of the ROS produced in mitochondria come from complex I. A

correlation between PD and complex I was observed when a reduction in the complex's activity in human brains lead to significant neuronal loss associated with PD⁸⁰. When monitoring NADH:ubiquinone oxidoreductase enzymatic activity of complex I, significant decreases were observed in PD frontal cortices, SNc and skeletal muscles, when compared to age-matched controls⁸¹. Additionally, signs of chronic oxidative stress, such as lipid peroxidation, nitration and reduction in glutathione levels, a major cellular antioxidant, were observed in the SNc as early markers of nigral loss in PD^{82,83}.

At present, it is still unclear whether mitochondrial dysfunction plays a causal role in PD or it only correlates with the progression of the disease. In any case mitochondrial dysfunction does play a key role in the physiological processes of PD⁸⁴, based on studies of familial PD genes⁸⁵, discussed in section 1.8.

Mitochondrial dysfunction and oxidative stress are also apparent when modeling familial PD with genes associated with autosomal recessive early-onset parkinsonism such as *DJ-1*, *PINK1* and *PRKN*. DJ-1 can protect cells against a rise in oxidative stress driven by complex I inhibitors or hydrogen peroxide (H₂O₂) treatments, which is abrogated by DJ-1 PD-linked mutations⁸⁶. Additionally, DJ-1 can act as antioxidant, through its direct oxidation at cysteine (C) 106⁸⁶; and in mice treated with MPTP, DJ-1 levels are increased in SNc mitochondrial fractions⁸⁷. In humans, PINK1 mutations result in mitochondrial respiratory chain dysfunctions⁸⁸; and loss of function of PINK1 generated a decrease in mitochondrial membrane potential and consequentially a rise in ROS production in both human and mouse DA neurons⁸⁹. Furthermore, PINK1 animal models develop

mitochondrial deficits, including changes in morphology, increased sensitivity to oxidative stress, and nigral cell loss^{90,91}. In addition, recent studies reported mitochondrial antigen presentation, which will be discussed further as it relates to parkin in section 1.10.9, and establishment of cytotoxic mitochondria-specific CD8⁺ T cells in the periphery and the brain of PINK1-null mice post intestinal infection with Gram-negative bacteria. This resulted in loss of striatal DA projections and motor impairments⁹².

In PD patients with *prkn* mutations, mitochondrial complex I activity is impaired⁹³, and in unbiased proteomic studies, *prkn*-null mice display alteration in abundance and expression of several proteins and enzymes involved in mitochondrial function, redox reactions and oxidative stress^{94,95}. Furthermore, overexpressing parkin in mice treated with MPTP reduced neuronal damage and loss, by protecting mitochondria from oxidative stress and reducing the levels of α -synuclein⁹⁶.

In addition, accumulation of WT α -synuclein induced DA neuron cell death by reducing mitochondrial complex I activity and increasing ROS production in mice⁹⁷, and its inclusions increased dendritic mitochondrial oxidative stress⁹⁸.

Some research points to the role of mitochondrial genome instability in PD. For instance, studies using cytoplasmic hybrid (cybrid) cells have reported deficits in mitochondrial DNA (mtDNA) in PD patients⁹⁹. Cybrids are produced by replacing the mtDNA of a healthy cell with one obtained from affected individuals. It has been shown that PD patient-generated cybrids display depolarized mitochondria, lowered ATP production, increased ROS release, and reduced complex I activity. This suggests that the deficiency in complex I observed in PD cases could be inherited from an alteration in the

mitochondrial genome⁹⁹. However, in the pathogenesis of PD, mtDNA deletions may represent a secondary rather than a primary event. Inducing a mutation (D257A) in the exonuclease domain II of DNA polymerase subunit gamma (POLG), resulted in inhibition of its proof reading activity and accumulation of mtDNA deletions¹⁰⁰. Nevertheless, POLG mice do not present with SNc DA cell loss¹⁰¹. However, when parkin deficiency is added, the POLG mouse displays signs of DA neuron cell loss, eluding to a role for parkin in regulating mitochondrial function and oxidative stress pathways¹⁰².

Taken together, the following graph⁵⁵ summarizes several of the physiological pathways and processes currently understood to be involved in PD (**Figure 1.3**). Several PD-related genes and proteins are implicated and displayed in the graphical depiction. They will be covered separately in section 1.8 on the genetics of PD.

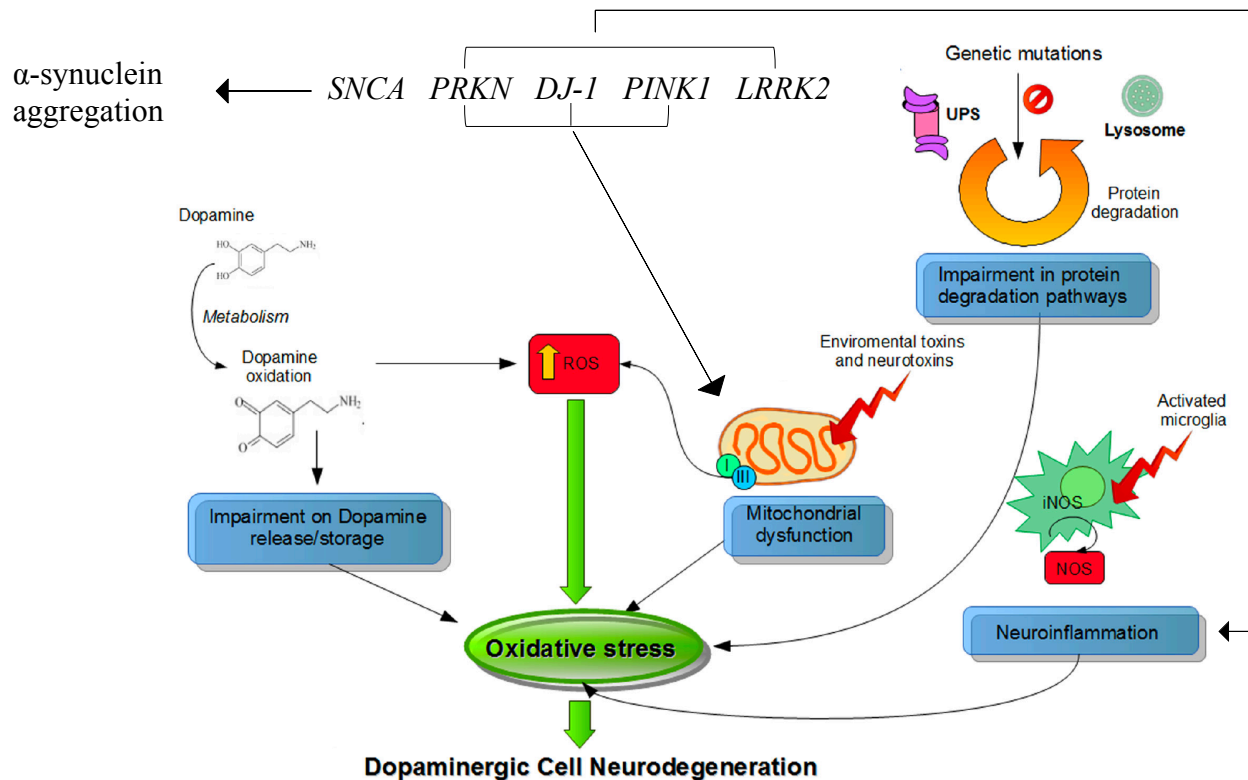


Figure 1.3: Pathophysiological processes of PD.

Several pathways and their dysfunctions have been suggested to be implicated in the pathogenesis of PD. Many of these mechanisms are regulated by PD-associated genes, discussed in section 1.8. Altered expression or mutation in these proteins could result in one, mitochondrial dysfunction, namely in complex I; two, protein aggregation and dysfunction in the UPS involved in degradation pathways; and three, neuroinflammation and activation of brain immune cells (microglia). Furthermore, the dopamine metabolism with SNc neurons generates reactive toxic species, dopamine quinones and ROS, that further induce protein aggregation, mitochondrial dysfunction and neuroinflammation. Modified from Blesa et al., 2015.

1.6 Biomarkers

The usefulness of PD biological markers rest on their capacity for predicting which individuals are at a higher risk of developing PD, as well as making it easier to monitor the progression of the condition and the role of medication in reducing the loss of dopaminergic neurons.

Biological markers are commonly used in the diagnosis and progression of neurodegenerative diseases, including PD. Cerebrospinal fluid (CSF) is an immunological and mechanical protector of the brain, as well as a source of brain-derived proteins. It has been suggested that a decrease in the levels of α -synuclein and DJ-1 in the CSF is a potential indicator of PD diagnosis but not severity¹⁰³⁻¹⁰⁵. Furthermore, a combination of CSF-derived biomarkers such as total tau, phosphorylated tau, amyloid beta peptide 1-42 ($A\beta(1-42)$), Flt3 ligand, and fractalkine could differentiate PD patients from healthy controls and patients with AD and multiple system atrophy (MSA). Particularly, Flt3 ligand measured in from CSF of PD patients, was able to differentiate between PD and MSA, which are known to clinically overlap, with 99% sensitivity and 95% specificity¹⁰⁶.

As previously mentioned, strong evidence implicates neuroinflammation in the pathogenesis of PD. Therefore, using CSF-derived inflammatory markers as a diagnostic tool has gained strong ground in recent years. For instance, C-reactive protein (CRP) levels were significantly increased in PD patients with a dementia diagnosis (PDD) when compared to age- and sex-matched non-demented PD patients and healthy controls. CRF

and monocyte chemotactic protein-1 (MCP-1) levels were associated with severe symptoms of depression and fatigue. CSF inflammatory markers may be used as indicators of disease severity and the progression of non-motor symptoms¹⁰⁷.

The combination of biomarkers and prediction modelling, mathematical interrogation and application of neuroimaging-based techniques^{108,109}, is crucial for early detection, monitoring of disease severity and progression, and highlighting factors for the development of better cause-directed therapies.

1.7 Therapies

At present, there are no available treatments that can stop, alter the course of disease or reverse the neurodegenerative process of typical PD. All related, properly controlled clinical trials have failed. However, with advancements in understanding the multi-layered pathogenesis of PD, treatments to manage symptoms and cause-directed therapies have been the topic of several studies in recent years.

1.7.1 Dopamine replacement therapy

Medications that can supplement the brain with dopamine were developed early on to help patients manage their condition more effectively. The treatment with Levodopa, a dopamine precursor (L-DOPA) and Carbidopa, which increase L-DOPA availability in the brain by inhibiting its peripheral breakdown, became a gold standard in the field^{17,110,111}. Even though the combination treatment does not halt disease progression, it

can provide patients with motor symptoms relief with little to no side effects, which can include dyskinesia and hallucinations.

Other treatments that target the decrease in dopamine availability due to the progressive loss of SNc DA neurons are dopamine agonists such as Pramipexole and Ropinirole, and preventing dopamine degradation with catechol-O-methyltransferase (COMT) inhibitors such as Entacapone^{112,113}.

1.7.2 Other therapies

Some suggested therapies seek to target the underlying biological factors that participate in the etiology of PD. For instance, some therapeutic approaches to target mitochondrial dysfunction in PD have been proposed. The largest clinical trial in this regard is the Deprenyl And Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP), which investigated the neuroprotective effects of a MAO-B inhibitor (selegiline) and vitamin E in patients with an early form of PD¹¹⁴. A more recent treatment proposal is Rasagiline, another MAO-B inhibitor. It is suggested to have stronger effects than selegiline and its metabolites may have antioxidant properties. In a clinical trial conducted two decades ago¹¹⁵, Rasagiline was shown to provide long-term improvements when used as an early treatment. However, a more recent study with a larger sample size confirmed earlier results at the 1 mg dose, but failed to replicate the induced improvements at the 2 mg dose¹¹⁶.

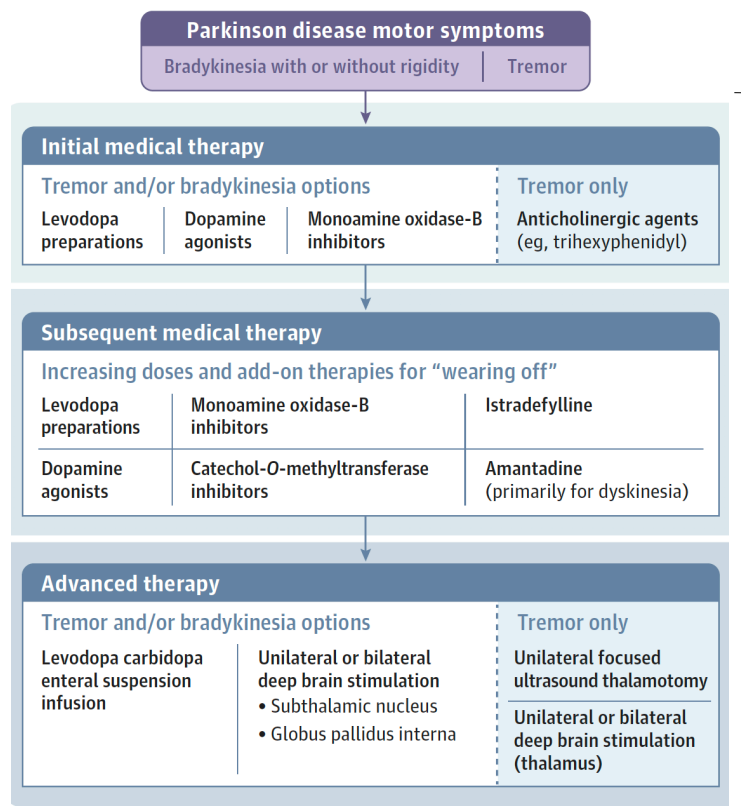
Surgical procedures have become more available in recent years; however, their use requires careful patient selection and screening. Deep brain stimulation (DBS) for

example focuses on the nigro-striatal pathway discussed in section 1.4.1. By inserting an electrode in the STN, the procedure mimics the pacemaking activity of the degenerating DA neurons in the SNc projecting to the striatum, and regulates basal ganglia circuitry functioning¹¹⁷. In a similar fashion, and based on an understanding of PD genetics and pathogenesis, adeno-associated virus (AAV)-mediated gene delivery can target the STN (and striatum) and alter basal ganglia activity, by affecting for example dopamine availability and gamma-aminobutyric acid (GABA) synthesis¹¹⁸.

Recent efforts have looked into stem cells as potential therapeutics for PD, utilizing induced pluripotent stem cells (iPSC) from PD patients to derive personalized dopamine progenitor cells. In a recent study by Schweitzer et al., patient specific progenitor cells were produced and characterized as having similar phenotypic properties as SNc DA neurons, in the absence of immunogenicity. Implanting these cells in the putamen in the left hemisphere followed by the right hemisphere six months apart, resulted in graft survival (without the use of immunosuppressors) and reduction or improvement in clinical measures of PD symptoms at 18 to 24 months¹¹⁹. A major concern still remains regarding the health of these cells with time, namely what would prevent them from degenerating? Will the CNS-wide process of disease be stopped by the engrafted iPSC transplants?

Other forms of treatments, such as physical therapy, are also recommended to improve mobility, overall fitness as well as mental health. Older patients can as well benefit from assist devices that can prevent falls and decrease episodes of freezing. The following

figure summarizes proposed general approaches to the treatment of mainly motor symptoms in PD patients¹²⁰ (**Figure 1.4**).



Rehabilitation therapy:

Including exercise, physical therapy, occupational therapy and speech therapy; for all symptoms and across all disease stages.

Figure 1.4: Proposed approaches to the treatment of mainly motor symptoms in PD patients.

Treatments that alter dopamine availability in the brain are used as initial therapies. These can be Levodopa, dopamine agonists and MAO-B inhibitors. In early-onset PD patients, experiencing motor symptoms with lack of cognitive decline, anticholinergic agents can be used to treat tremor. Subsequent medical interventions are required as the disease progresses, including increases in dosages, and the addition of other therapies with complementary mechanisms of action, such as COMT inhibitors. When PD patients begin to experience medication-induced dyskinesias, and worsening symptoms or functional impairments as the medication dose wears off, advanced therapies can be utilized, namely deep brain stimulation. Modified from Armstrong et al., 2020.

1.8 Genetics of PD

Since the mutation in the *SNCA* gene was identified to cause PD in 1997²³, four other genes have been further characterized to be linked to PD and have been the topic of extensive studies. In the past two decades, several other genetic linkages that appear to play a role in PD or confer a risk have also been identified. Genetic contribution accounts for approximately 5-10% of all PD cases, and these genes range from risk factors that confer susceptibility to developing PD with moderate to small effect sizes, such as *GBA*, to highly penetrant monogenic forms, where a mutation in the gene is sufficient to cause the disease¹³. Mutations in the *PARK1/4* or *SNCA* and *PARK8* or (leucine rich repeat kinase 2) *LRRK2* genes result in autosomal dominant forms of PD, whereby mutations in the *PARK2* or *PRKN*, *PARK6* or *PINK1*, and *PARK7* or *DJ-1* result in autosomal recessive forms of PD (ARPD). Special consideration should be given to the PARK nomenclature of loci. It was initially used in the characterization of familial cases of PD where chromosomal regions were mapped but the causative genes were still unknown¹²¹. Once genes were identified, the locus symbols were replaced by gene names. This information is particularly important when using data from studies published at different times.

1.8.1 Autosomal dominant forms

SNCA and *LRRK2* are the two genes most studied in autosomal dominant forms. These PD cases are broadly characterized as late-onset featuring motor deficits, Lewy body pathology and cognitive decline.

1.8.1.1 *SNCA* (*PARK1/4*)

As mentioned previously, *SNCA*, coding for the α -synuclein protein, was the first gene to be identified casually link to PD²³. The literature describes several causative genetic variants, including point mutations A53T²³, A30P¹²², E46K¹²³ and G51D¹²⁴. However, duplication¹²⁵ and triplication¹²⁶ events are much more common, with an earlier age of onset and more pronounced non-motor symptoms¹²⁷. It is suggested that these variants of *SNCA*, increase the ability of the protein to oligomerize, aggregate and cause cell death^{128,129}. The function of α -synuclein that renders it essential to DA neurons is currently still unknown; however, it has been characterized as a pre-synaptic protein involved in dopamine transmission¹³⁰. Its pathogenicity arises from its propensity to aggregate into toxic oligomers, leading to β -sheets formation¹³¹, as well as its fibril species being identified as the main component of LBs¹³². It has been suggested that the formation of Lewy bodies is a cellular response to quench toxic oligomers and promote neuroprotection¹³³.

1.8.1.2 *LRRK2* (*PARK8*)

Mutations in *LRRK2* are the most common in autosomal dominant forms, as well as being found in idiopathic cases. *LRRK2* PD-linked mutations were first described in 2004¹³⁴⁻¹³⁶. The most common mutation in *LRRK2* is G2019S, which is found in 1% of sporadic PD and 4% of familial PD, and results in a significant increase in the protein's kinase activity¹³⁷. However, the mutation does not demonstrate full penetrance, which suggests that other contributors are important for disease genesis and progression¹³⁶. From a clinical perspective, *LRRK2*-linked PD is usually undistinguishable from

idiopathic PD, with motor symptoms that are tremor-dominant and levodopa-responsive at the onset, and seem to be less likely to present with hyposmia and cognitive changes¹³⁸. The *LRRK2* gene encodes a large, > 250kDa, protein containing a kinase domain, a GTPase domain, and other protein-protein interaction domain. The most common point mutations are localized to the kinase domain (G2019S, and I2020T); however, some PD-linked mutations are also found in the Ras of Complex (ROC) domain (R1441C), the C-terminal of ROC (COR) domain (Y1699C) and the Leucine Rich Repeat (LRR) domain (I1122V)¹³⁹. The physiological function of LRRK2 in PD remains heavily debated. It has been suggested that its kinase activity mediates most of its toxicity¹⁴⁰, and it has been predominantly studied in the context of a neuronal kinase¹⁴¹. It has been strongly linked to the regulation of autophagy¹⁴². Our own research has shown that it plays a key role in the innate immune system⁶⁸⁻⁷⁰, something we hypothesize is directly linked to its association with PD.

In recent years, mutations in the vacuolar protein sorting-associated protein 35 (VPS35) were linked to autosomal dominant forms of PD, with D620N being the most common¹⁴³. VPS35 is a component of the retromer complex involved in the transport of proteins from endosomes to the trans-Golgi network^{144,145}. This suggest that vesicle and endosomal trafficking is implicated in the pathogenesis of PD. However, further research is needed to validate this theory.

1.8.2 Autosomal recessive forms

Several genes have been identified to cause autosomal recessive forms of PD; however, *PRKN*, *PINK1*, and *DJ-1* have been the most widely researched. While the three genes encode for three different proteins with speculated different functions, they share similar pathological features. An early age of onset is common in mutations in these genes, along with mainly motor symptoms, and absence of Lewy body pathology, at least for *PRKN* patients¹⁴⁶. This yet again points to the heterogeneity of PD and the different clinical and pathological forms of the disease. Autosomal recessive cases present with the same loss of SNc DA neurons, however the pathways leading to it may be different. The stratification of PD cases is very crucial in regard to developing appropriate therapies.

Given that parkin is the subject of this dissertation, it will be discussed in detail, in section 1.10.

1.8.2.1 *PINK1 (PARK6)*

Phosphate and tensin homolog (PTEN)-induced serine/threonine kinase 1 (PINK1) was first identified in 2001¹⁴⁷, and was found to be associated with early-onset PD that same year¹⁴⁸. PINK1 subcellularly localizes to the mitochondria, given its mitochondrial targeting signal (MTS), and hence a function in regulating mitochondrial health has been most widely associated with the protein and studied. This role has been validated in several animal models, whereby a PINK1-deficiency resulted in mitochondrial disruptions, ultimately leading to DA neuron degeneration, in flies¹⁴⁹. An interplay between PINK1 and parkin has also been the subject of many studies, establishing a role

for the interaction in mitochondrial quality control and the regulation of mitophagy, which will be further discussed in the context of parkin^{150,151} G309D and W437X are two PINK1 homozygous mutations found in three Italian families, and have been found to disrupt the protein's kinase activity^{152,153}.

1.8.2.2 DJ-1 (*PARK7*)

A link between DJ-1 mutations and early-onset PD was first identified in 2003¹⁵⁴. Prior to this discovery, DJ-1 was thought to be an oncogene¹⁵⁵. Mutation in DJ-1 only account for 1-2% of cases¹⁵⁶, and patients present with phenotypes similar to PINK1 and parkin-associated PD¹⁵⁷. The DJ-1 gene encodes a ubiquitously expressed protein that is suggested to be an oxidative chaperone¹⁵⁸. The most common PD-linked mutation L166P is thought to render the protein unstable leading to its 20S proteasome-mediated degradation¹⁵⁸. Other genetic variants, including, frameshifts and missense mutations, are also thought to induce protein instability¹⁵⁹. Given its reactive cysteine residue, C106, it has been described as a cellular antioxidant with an oxidative stress-reducing function¹⁶⁰

1.8.3 PD-associated genes

GBA has been the subject of many studies in recent years, and has emerged as one of the strongest PD-associated gene, and the most common genetic risk factor¹⁶¹. GBA localizes to the lysosome where it associates with the lysosomal membrane¹⁶². It possesses a glucosylceramidase activity allowing it to hydrolyse the cleavage of glucocerebroside that is abundant in cell membranes and involved in glycolipid metabolism¹⁶³. Homozygous mutation in GBA results in Gaucher's disease, a lysosomal storage

disorder¹⁶⁴. The link between GBA and PD became evident when Gaucher's disease patients presented with signs of parkinsonism, and pathological studies showed the presence of Lewy bodies¹⁶⁵. Heterozygous mutation carriers have on average a 15% increased risk of developing PD¹⁶⁶, with some cases up to 31%¹⁶⁷. Clinically these patients present with an earlier age of onset and more frequent dementia¹⁶⁸. As of 2020, the presence of GBA mutation is a major predictor of cognitive decline¹⁶⁹.

The following table adapted from Lunati et al.¹³ summarizes the PD- associated genes and their phenotypes (**Table 1.1**).

Gene	Map position	Type of mutations	Inheritance	Disease onset	Clinical phenotype	Features	Response to levodopa	Neuropathology
Autosomal dominant (AD)								
LRRK2	12q12	p.Gly2019Ser, the most common	AD/AR	Late	Typical	Phenotype less severe than IPD	+	LB, loss of DA neurons, +/- LB, +/- Tau pathology
SNCA	4q22.1	p.Ala53Thr and duplications. p.Ala30Pro p.Glu46Lys	AD	Early	Typical	Dementia	+	LB, LN, loss of DA neurons
			AD	Late	Atypical	Dementia and cerebellar sign	+	LB, LN, loss of DA neurons
			AD	Late	Typical	Frequent dementia and hallucinations	+	LB, LN, loss of DA neurons
			AD	Early	Atypical	Pyramidal signs	+/-	LB, LN, loss of DA neurons
VPS35	16q11.2	p.Gly51Asp p.Ala53Glu Tripletations	AD	Early	Atypical	Pyramidal signs, myoclonus	+	LB, LN, loss of DA neurons
			AD	Early	Typical	Frequent dementia	+	LB, LN, loss of DA neurons
			AD	Early	Typical		+	undetermined
ATXN2	12q24.12	Interrupted CAG repeat expansions	AD	Early	Typical	No dementia	+	LB, loss of DA neurons
GCH1	14q22.2		AD	Early	Typical	Long-term motor complications ± dystonia	+	LB, loss of DA neurons
Autosomal recessive								
PRKN	6q26		AR	Juvenile or early	Typical		+	Loss of DA neurons, very few LB
PINK1	1p36.12		AR	Juvenile or early	Typical		+	LB, loss of DA neurons
DJ1	1p36.13		AR	Juvenile or early	Typical		+	LB, loss of DA neurons
ATP13A2			AR	Juvenile	Atypical	Dementia, pyramidal signs, supranuclear vertical gaze palsy, early motor complications	+	ND
PLA2G6	22q13.1		AR	Juvenile or early	Atypical	Dementia, pyramidal signs, ataxia, psychiatric features, ocular disorders, early motor complications	+	LB, LN
FBXO7	22q12.3		AR	Juvenile	Atypical	Pyramidal signs, psychiatric or motor complications	+	ND
Risk factor								
GBA	1q22		AD	Early or late	Typical	Frequent dementia	+	LB, loss of DA neurons

Table 1.1: PD-linked genes and risk factors.

A table summarizing autosomal dominant and autosomal recessive PD-associated genes, risk factors, and their respective phenotypes. IPD, idiopathic PD; juvenile, age at onset 20 years; early, age at onset between 21 and 50 years; late, age at onset > 50 years; response to levodopa, +: good response, +/-: moderate response, -: poor response; LB, Lewy bodies; LN, Lewy neurites; and ND, no data reported. Modified from Lunati et al., 2018.

1.9 Modelling PD

To better understand disease genesis and progression, and in order to test and evaluate therapies, several *in vivo* models of PD have been developed, based on an understanding of the hallmarks, genetics and environmental contributors of PD.

1.9.1 *In vivo* toxin models

In an attempt to recreate PD-like phenotypes in animal models, akin to human patients, and to better understand mechanisms of disease, several toxin-based models were developed, including 6-OHDA, MPTP, rotenone and paraquat¹⁷⁰.

1.9.1.1 6-OHDA

6-OHDA was the first toxin to be reported to affect the nigrostriatal pathway¹⁷¹. It does not cross the blood brain barrier (BBB) and for that reason it is most widely injected unilaterally to the SNc, striatum or medial forebrain bundle. 6-OHDA accumulates in the cytosol and induces mitochondrial dysfunction, a rise in oxidative stress and up to 60% cell loss in TH-positive neurons¹⁷². Treatment with 6-OHDA also results in behavioural deficits, namely rotational behaviour after apomorphine injection^{173,174}.

1.9.1.2 MPTP

In 1983, while injecting opioids contaminated with MPTP, human drug users developed typical signs of PD. Since then, MPTP became a gold standard in PD modelling to induce hallmarks of PD, namely SNc DA neurons loss accompanied with behavioural abnormalities. This also reinforces the hypothesis of environmental (in this case

neurotoxin) contributions in PD pathogenesis¹⁷⁵. Chronic MPTP-induced damage is specific to DA neurons, whereby it crosses the BBB, is metabolized to MPP⁺ via MAO-B in glial cells and transported into neurons via the dopamine transporter (DAT)¹⁷⁶. When it reaches DA neurons, similarly to 6-OHDA, it causes mitochondrial dysfunction in complex I, a rise in ROS production, and cell death¹⁷⁷. In some cases Lewy body inclusions are observed¹⁷⁸. With significant DA cell loss motor impairments become apparent¹⁷⁶.

1.9.1.3 Paraquat

N,N'-dimethyl-4,4'-bipyridiniumdichloride (Paraquat) is an agriculture herbicide with a similar structure to MPP⁺. Injection of paraquat in animals results in similar changes seen with MPTP. It induces dysfunction in the redox cycle of mitochondria, and increase in oxidative stress causing lipid, DNA and protein damage^{179,180}. SNc DA neuronal loss in paraquat models is dose-dependent, and propagated through DAT similarly to MPTP¹⁸¹, and includes Lewy body formation in both mice and rats¹⁸². Furthermore, some paraquat animal models have been used in conjunction with 2-(dithiocarboxy)aminoethylcarbamodithioato(2-)-kS, kS' manganese (Maneb). It is a well-known fungicide with the ability of potentiating the damaging effects of both MPTP and paraquat¹⁸³. Maneb treatments alone have also been shown to cause decreased motor activity and SNc DA neurons loss¹⁸⁴.

1.9.1.4 Rotenone

Rotenone, naturally occurring in certain plants, is a herbicide and a pesticide with neurotoxic effects¹⁸⁵. Due to its lipophilic properties, post intravenous (IV) injections, it can cross the BBB and result in complex I-mediate mitochondrial defects leading to DA neuronal loss, as well as α -synuclein aggregation and Lewy body inclusions¹⁸⁶. The effects of rotenone are widespread and not exclusive to dopaminergic cells, as it can extend to serotonergic, noradrenergic, and cholinergic systems¹⁸⁷. Behavioural deficits have also been reported in rotenone-based animal models¹⁷⁵. However, rotenone-based animals models are seemingly hard to replicate due to the high mortality rates observed in some rodents¹⁸⁸.

The following graph is adapted from Jagmag et al.¹⁸⁹, and summarizes the major toxin models (**Figure 1.5**).

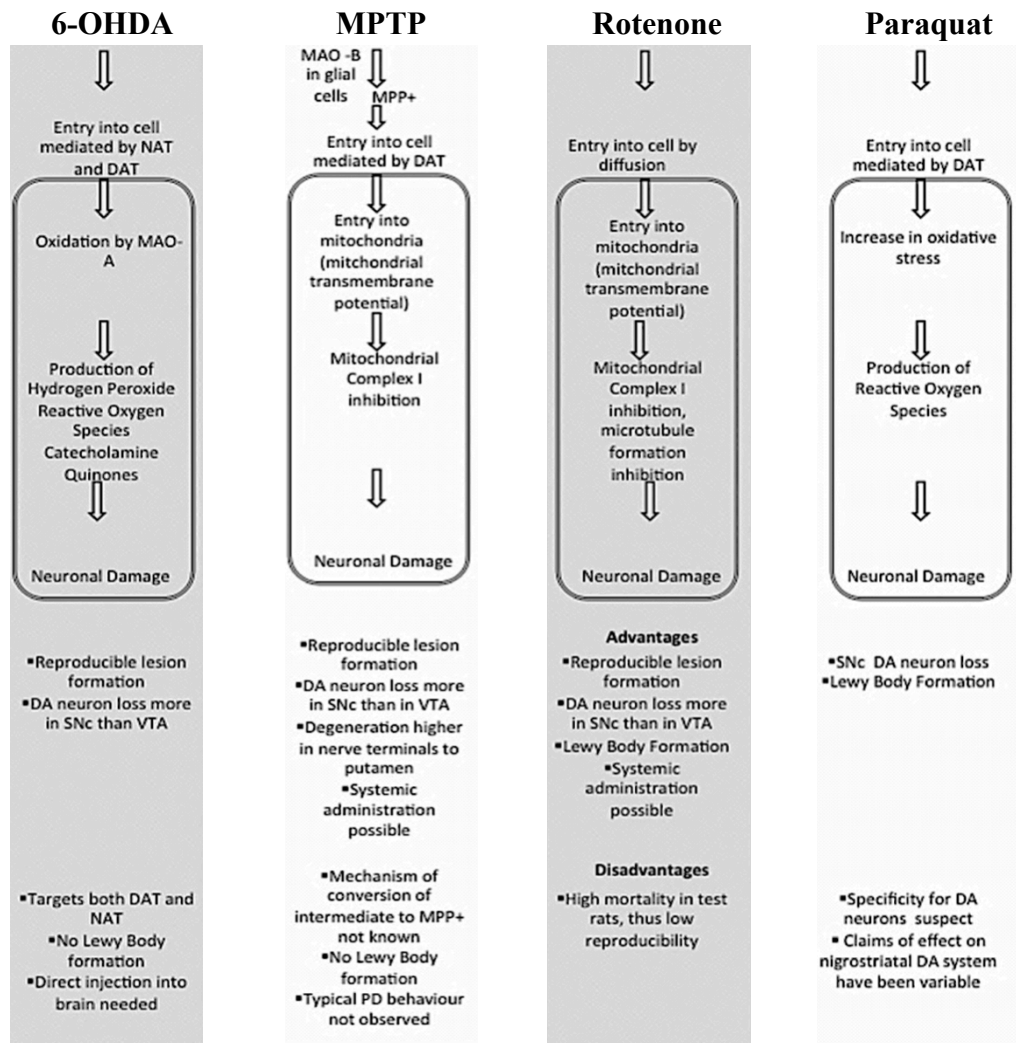


Figure 1.5: The major *in vivo* toxin-based models.

A comparison between the four main toxin-based models, 6-OHDA, MPTP, rotenone, and paraquat, used to induce parkinsonian phenotypes, including the advantages and disadvantages of each one. NAT, noradrenergic transporter; and VMAT2, vesicular monoamine transporter 2. Modified from Jagmag et al., 2016.

1.9.2 *In vivo* genetic models

Based on our current understanding of familial cases of PD, genetically-modified animal models have been developed with goals to: one, better understand the signaling pathways associated with known causal genes; two, recapitulate the cellular, biochemical and motor phenotypes observed in PD patients; and three, allow for therapeutic screens.

SNCA, *LRRK2*, *PARKIN*, *DJ-1* and *PINK1* are the five genes most frequently targeted as disease models for PD, as they all have been linked to familial cases. What these genetic models could offer is a better understanding of whether the loci associated are the main drivers, carriers or helpers of disease progression, and the discovery of new disease mechanisms¹⁸⁹. Murine or mouse models are most frequently used as animal-based modelling of PD pathogenesis. The ease in breeding strategies, and colony maintenance, along with conserved gene homology between mice and human, and similarities in anatomical, physiological, and behavioural aspects makes them the number one choice. In the following table (**Table 1.2**) adapted from Jagmag et al.¹⁸⁹, the major mouse models based on the aforementioned four genes are summarized, with the exception of parkin, which will be covered in section 1.10.10.

	Types	Promoter	SN neuron loss	Inclusion bodies	Motor impairment
α-synuclein	WT	PDGF- β	–	+	+
	WT/A53T	Thy1	–	+	+
	WT/A30P/A53T	TH	–	–	ND
	WT	Prp	–	–	–
	A53T	Prp	–	+	+
	WT	Prp	–	–	–
	A30P	Prp	–	+	–
	A53T	Prp	–	+	+
	WT	TH	–	–	–
	A53T and A30P	TH	+	–	+
	A30P	PrP	–	+	+
	WT/A30P/A53T	CMV	+	+	+
	WT (1–120)	TH	–	+	+
	WT	CMV	+	–	ND
	WT	CaMKII	+	–	+
	WT (1–130)	TH	+	–	+
	WT (1–119)	ROSA26	–	–	ND
	A53T	ROSA26	–	–	ND
	E46K	ROSA26	–	–	ND
	A53T	Pitx3	+	–	+
	WT/A53T	CMVE- Syn 1	+	+	+
LRRK2	R1441C/G*	mLRRK2	ND	ND	ND
	R1441C*	mLRRK2	–	–	–
	WT [#]	mLRRK2	–	–	+
	R1441G [#]	mLRRK2	–	–	+
	WT [#]	hLRRK2	–	–	–
	G2019S [#]	hLRRK2	–	–	–
	exon 1*	mLRRK2	–	+	ND
	exon 29, 30*	mLRRK2	–	+	ND
	G2019S [#]	CMVE- PDGF β	+(DA loss)	–	–
	R1441C [#]	CMVE-PDGF β	–	–	+
	WT [#]	CMVE-PDGF β	–	–	–
	G2019S [#]	CMVE-PDGF β	+(DA loss)	–	+
	R1441C [#]	ROSA26	–	–	–
PINK1	4–7 Exon mutation	–	–	–	–
DJ-1	Exon 2 deletion	–	–	–	+
	Exon 2 deletion	–	–	ND	ND
	Exon 3–5 deletion	–	–	ND	–
	Exon 7 inactivation	–	–	ND	+
	Exon 2–3 deletion	–	–	ND	–
	Exon 2 deletion	–	–	–	+
	Exon 1 stop	–	+	ND	–

Table 1.2: Genomic mouse models of PD.

Summary table of mouse models of autosomal dominant (α -synuclein and LRRK2), and autosomal recessive (PINK1 and DJ-1) PD.

Table 1.2: Continued-

WT, wild-type / human α -synuclein; PDGF- β , platelet derived growth factor- β ; Thy-1, Thy-1 cell surface antigen; TH, tyrosine hydroxylase; Prp, prion protein promoter; CMV, cytomegalovirus; CaMKII, calcium/calmodulin-dependent protein kinase II; ROSA26, ROSA β geo26P locus; Pitx3, paired-like homeodomain3; CMVE-Syn1, cytomegalovirus enhanced synapsin1; mLRRK2, murine LRRK2; hLRRK2, humanLRRK2; *mouse, #human; CMVE-PDGF β , cytomegalovirus enhanced-platelet derived growth factor- β ; and ND, no data reported. Modified from Jagmag et al., 2016.

Invertebrate models have also been utilized in the modelling of PD, and served as valuable tools in understanding the fundamental processes underlying SNc DA cell loss in patients. These models have also been widely used as screening tools to shed more light on key players in PD-associated neurodegeneration, and ultimately expand their used to more clinically relevant model organisms. *Saccharomyces cerevisiae*, the common budding yeast, *Caenorhabditis elegans*, a nematode, and *Drosophila melanogaster*, the common fruit fly, are some of those organisms. *Rattus norvegicus* (rat) and *Drosophila melanogaster* (fruit fly) genetic PD models are summarized in the table found in the appendix section (**Appendix II**).

1.10 Parkin

In diagnosing neurological diseases, lesion localization within the nervous system traditionally took priority followed by elucidation of specific etiology. Hence, patients presenting with parkinsonism, as opposed to sporadic or idiopathic forms of PD, indicates a basal ganglia disorder, of which PD is one of several potential causes¹². A number of Mendelian forms of parkinsonism have been identified over the past two decade, with varied clinical and pathological overlap with PD. EOPD, previously referred to as autosomal recessive juvenile parkinsonism (ARJP), of which the most studied and most common cause is the loss of function of parkin, best exemplifies this class of disorders^{190,191}. Prior to the identification of parkin and other genes involved in autosomal forms of PD, EOPD was characterized by: one, the existence of affected family members within the same generation (in many cases born to consanguineous parents); two, an age

of onset before 40 years; three, an atypical parkinsonian phenotype; and four, the absence of LB pathology in the brain.

1.10.1 Parkin-linked parkinsonism

The gene locus *PARK2* of EOPD was first mapped in 1997 to chromosome 6q25.2–27¹⁹². In 1998, the first evidence for a point mutation in the *PRKN* gene in a Japanese family that linked to autosomal recessive PD and resulted in an early-onset development of the disease, was published by Kitada et al.¹⁹³. Further studies confirmed the genetic linkage between *PRKN* and EOPD in patients of European and North African descent¹⁹⁴. Mutations in the *PRKN* gene are the most common cause of EOPD, with >100-PD associated variants identified to date¹⁹⁵. It is thought that these variants account for 10 to 20% of all EOPD cases, and have so far been identified in numerous families of different ethnic backgrounds²⁰. Parkin mutations are spread out throughout the gene and affect all 12 exons. They range from small deletions to changes in thousands of nucleotides, believed to cause a loss in parkin functionality.

Parkin-linked PD diverges from both idiopathic PD and other genetic forms of PD by a number of criteria, which suggests that even though the end result of DA cell loss is common to all forms, parkin-associated PD may be a distinct disease entity with different pathological pathways. Similarly to EOPD, the features include: one, a mean age of onset around 40 years¹⁹⁶; two, atypical PD phenotypes, including symmetric onset of motor deficits, early dystonia, reflex changes, diurnal fluctuations in symptoms and an overall slow progress of the disease¹⁹⁷; three, the absence of non-motor manifestations, including

cognitive decline, dementia and hyposmia (suggesting a more restricted pathology, which will be discussed in the next section)¹⁹⁸; and four, the absence of LBs^{199,200}, even though cases with LB pathology have been described^{201,202}.

1.10.2 Clinical manifestations of parkin-associated EOPD

As mentioned previously, parkin-associated PD is traditionally characterized by a recessive inheritance pattern, accompanied by typical PD motor symptoms, including bradykinesia, rigidity and resting tremor. Furthermore, with the earlier age of onset, patients present with dystonia localized to the foot, and hyperreflexia in the lower limbs. Additionally, patients with parkin mutations experience a slower disease progression when compared to idiopathic or sporadic PD, respond well to dopamine replacement therapy, namely Levodopa, however they develop an earlier onset of levodopa-induced dyskinesias²⁰³. With further studies, it became apparent that distinguishing parkin patients solely based on their clinical features was more challenging^{196,204}. For instance, clinical manifestations such as dystonia and hyperreflexia, known to be more frequent in EOPD patients, are less frequent than previously reported, making it more difficult to separate EOPD cases from idiopathic ones²⁰⁵. When analysing a larger number of families, the age of onset ranged from 7 to 58 years, with an average of 38 years. Even within the same family where members carry the same parkin mutation, variation up to 20 years in the age of onset was observed¹⁹⁶. This points at other contributing or modulating factors and highlights the adoption of the EOPD nomenclature over ARJP. Furthermore, when assessing specific parkin mutations, there seem to be no correlation between the types of variants and specific clinical manifestations. For instance, missense mutations, which are

expected to interfere less with the protein's function, do not cause earlier age of onset or more severe phenotype than truncating mutations¹⁹⁶.

1.10.3 Pathology of parkin-associated EOPD

From histopathological studies, EOPD due to parkin mutations has been characterized by a loss of pigmented DA neurons in the SNc and LC, along with the absence of LB pathology in the brain. The remaining surviving neurons show reduced content of intracellular pigmentation^{200,206-210}. Of note, some EOPD cases due to parkin mutations display features of astrocytic gliosis^{210,211}. The restriction of the disease solely to the two aforementioned areas without progression into the cortex, and the absence of widespread α -synuclein and ubiquitin positive LB formation^{199,212} could explain the clinical presentation of parkin-deficient EOPD, whereby patients present with motor symptoms in the absence of cognitive decline²¹³. This also highlights the heterogeneity of PD in general, and that cell death pathways in parkin-associated EOPD may be different than idiopathic or sporadic PD.

1.10.4 The *PRKN* (*PARK2*) gene

Ranking amongst the largest genes in the human genome, *PRKN* contains 12 exons separated by extended intronic regions, and spans more than 1.53Mb^{193,214}. The gene codes for a 465 amino acid (aa) long protein in humans, with a molecular weight of 52 kDa. The *PRKN* gene is evolutionary conserved and has orthologs in several species, including *C. elegans* (nematode), *D. melanogaster* (fruit fly), *R. norvegicus* (rat), and *M. musculus* (mouse)²¹⁵⁻²¹⁸. Of interest, the parkin protein is 464aa long in mice. The *PRKN*

promoter interestingly works bidirectionally, whereby it regulates the transcription of *PRKN*, as well as a gene upstream of and antisense to *PRKN*. The gene was named parkin co-regulated gene (*PACRG*), containing five exons and spanning 0.6 Mb²¹⁹. However, the physiological relevance of the gene to PD is still poorly understood. The parkin protein consists of an ubiquitin-like domain (Ubl) at its N-terminus, followed by four zinc-coordinating Really Interesting New Gene (RING) domains: RING0, RING1, in-between-RING (IBR) and RING2. Of note, RING0 is a fairly recent discovery and addition to parkin's architecture²²⁰.

1.10.5 EOPD-linked parkin mutations

The first mutations in parkin to be linked to EOPD were homozygous deletions affecting exons 3, 4, 5, 6 or J-17¹⁹³. Several other mutations have been identified since, including deletion of a single or multiple exons, duplication or triplications, and point or frameshift mutations. The latter can be further subdivided into missense mutations resulting in the replacement of an aa with another, nonsense mutations resulting in a stop codon and splice site mutations affecting the intronic regions^{196,205,221}. More than 120 pathogenic mutations have been identified with clusters in the RING domains owing to their critical functions²²², which will be discussed in section 1.10.7.4. However, it is worthy to note there is no mutational hotspot within parkin, as the mutations are spread throughout the protein, including the Ubl and linker regions. The parkin mutations associated with EOPD are homozygous or compound heterozygous, meaning with different mutations on each allele. As noted before homozygous mutations in parkin result in EOPD in the absence of LB pathology, however cases of compound heterozygous mutations with LB

pathology have been reported²⁰¹. These include a 40 base pair (bp) deletion in exon 3 on one allele and a R275W missense mutation on the other. Furthermore, published reports have identified cases with only one allele affected, and reduced penetrance^{201,221}, which could constitute a risk factor for EOPD, or even late onset PD. Homozygous or compound heterozygous mutations in parkin have a high penetrance (100%), whereas heterozygous mutations have a lower penetrance (1-25%)²²³.

The following graphical depiction represents parkin's domain architecture with selected EOPD-linked point mutations²²⁴ (**Figure 1.6**).

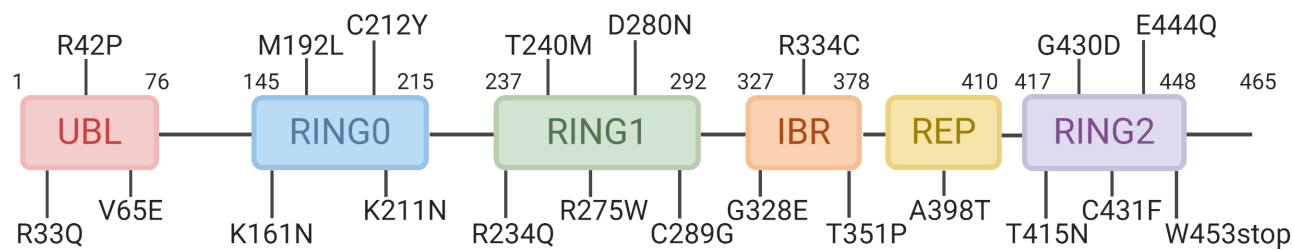


Figure 1.6: The parkin protein's structure and PD-linked point mutations.

A graphical depiction of human parkin's domain architecture, including the Ubl and the four RING domains. Selected PD-associated mutations are displayed, indicating the absence of a mutational hotspot across the protein. The effects of each mutation on the protein's structure and function, and their relevance to PD are summarized in the **Appendix III** table. Created with Biorender.com; modified from Seirafi et al., 2015.

1.10.6 The parkin protein

Parkin is predominately a cytosolic protein with strong expression in high energy-demanding tissues, including brain, heart, skeletal muscle and testes¹⁹³. Over the past two decades, several studies, revealed much about parkin's structure, and its E3 ubiquitin ligase function, discussed in the following section. At its N-terminus is an Ubl domain that is involved in substrate recognition, binding ubiquitin interacting motifs (UIM) and SH3 domains, association with the proteasome, and regulation of the protein's activity and cellular levels²²⁵⁻²³⁰. High-resolution crystallography studies of parkin fragments containing its RING domains, as well as low-resolution crystallography studies of the full-length protein revealed a compact arrangement, stabilized by hydrophobic interactions²³¹⁻²³³. The protein is coiled onto itself, whereby the N-terminal Ubl domain binds to the RING1 domain through an isoleucine (I)44-mediated hydrophobic surface, and RING0 is tightly associated with the C-terminal catalytic domain. Each RING domain coordinates two zinc ions through one histidine and seven cysteine residues, totalling to eight zinc ions per parkin protein. The collective RING1-IBR-RING2 domains are referred to as RING-between-RING (RBR)^{220,234,235}. The zinc ions are essential for structural stability of the protein and involved in the proper regulation of its activity²³⁶. As mentioned earlier, based on parkin's structure, it has been associated with an E3 ubiquitin ligase function. Its RING1 domain adopts a cross-brace zinc coordination topology that is similar to other RING-type E3 ligases. Additionally, crystal structures of phospho-parkin bound to an E2 enzyme (UbcH7)²³⁷, and HOIP in complex with a ubiquitin-charged E2²³⁸ confirm that the RING1 domain is the site of E2 enzyme binding. This observation was further characterized through NMR, molecular modelling

and mutagenesis studies, and identified the E2 binding site on parkin as an α -helix (residues 263 to 271) and two loops (residues 239 to 244, and 290 to 292). In contrast, RING0 and RING2 were identified as part of parkin's RING domains based on their primary amino acid sequences rather than their topology, as they differ from a classical RING formation. The RING2 domain is the site of parkin's catalytic activity, centering around residue C431. While the IBR domain is conserved among RBR ligases, RING0 is unique to parkin, with both domains' functions yet to be determined^{220,234}. The protein also contains two interdomain linker regions not resolved in crystal structures. The first separates the Ubl from the RING0, containing poorly conserved residues, including the human specific C95. The second linker separates the IBR from RING2, and is comprised of a conserved α -helix (residues 391 to 403) that binds to RING1. The α -helix within this linker region has been termed repressor element of parkin (REP) based on its role in regulating the protein's activity^{220,229,231,233-235}.

The following graphical representation models the parkin protein's domains, and its binding to an E2 ubiquitin-conjugation enzyme²²⁴ (**Figure 1.7**).

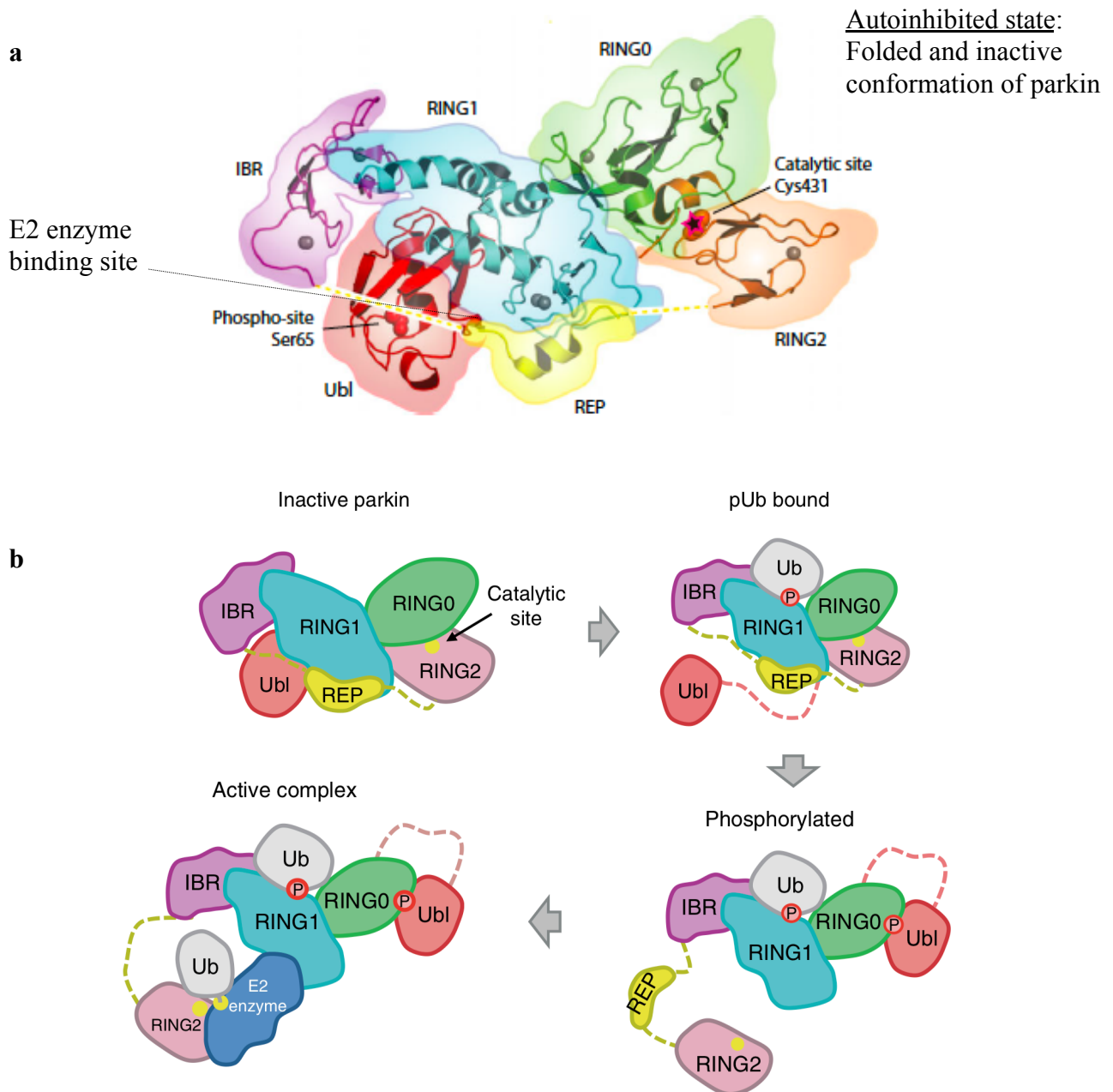


Figure 1.7: Crystal structure of Parkin.

Graphical representation of parkin highlighting **a**, the coiled inactive conformation of the protein with the Ubl domain and REP linker obstructing the E2 conjugating enzyme's binding site on RING1, as well as Ser65 the PINK1-dependent phosphorylation site inducing parkin activation; and **b**, sequential activation of parkin through binding of phospho-ubiquitin and PINK1-dependent phosphorylation at Ser65. Modified from Seirafi et al., 2015, and Sauvé et al., 2018. .

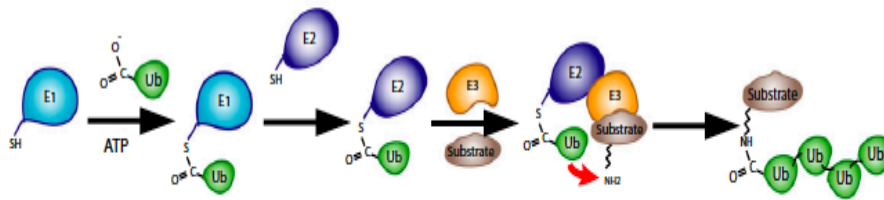
1.10.7 E3 ubiquitin ligase function

1.10.7.1 Parkin: a RING-HECT hybrid of the RBR family of E3 ligases

The ubiquitination process consists of the covalent attachment of monoubiquitin or polyubiquitin chains to lysine residues or the N-terminal amino group of substrates. This post-translation modification identifies proteins for degradation via the proteasome²³⁹. Additionally, ubiquitination can induce degradation through lysosome, signal for autophagy, and affect substrate activity or location^{240,241}. Ubiquitination is characterized by the sequential action of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. Ubiquitin contains seven lysine (K) residues and an N-terminal amino group on which polyubiquitin chains can be built. The E1 enzyme activates ubiquitin in an ATP-dependent manner, by forming a thioester bond between its catalytic cysteine and the C-terminal carboxyl group of ubiquitin, to be then conjugated to the catalytic cysteine of E2. The final step consists of the interaction between the ubiquitin-E2 complex and an E3 ubiquitin ligase, and the transfer of the ubiquitin onto an amino group of a substrate protein. The most common ubiquitin chain types are K48 and K63. The specificity of the ubiquitination process is generated by the E3 enzymes, as they generally recognize specific substrates²⁴². Three classes of E3 ligases exist, based on their structure and chemistry. RING-type, are characterized by the presence of a canonical C3HC4-type RING domain (RING1 of parkin) and facilitate the transfer of ubiquitin from the E2 enzyme to the substrate. Homologous to the E6-AP carboxyl terminus (HECT)-type contain a catalytic cysteine that receives the ubiquitin from the E2-conjugating enzyme and transfers it to the substrate. Lastly, RING-HECT

hybrid combines the structure of RING-type and the chemistry of HECT-type²⁴³. The RBR family of E3 ubiquitin ligases, to which parkin belongs, is considered a RING-HECT hybrid^{244,245}. In the following graph, the ubiquitination process is illustrated, along with the three-types of E3 ligases²²⁴ (**Figure 1.8**).

a



E3-mediated ubiquitination:

- Mono – Endocytosis.
- Linear poly – NF-κB signaling.
- K48 poly – Proteasomal and lysosomal degradation.
- K63 – Signaling pathways, DNA repair and lysosomal degradation.

b

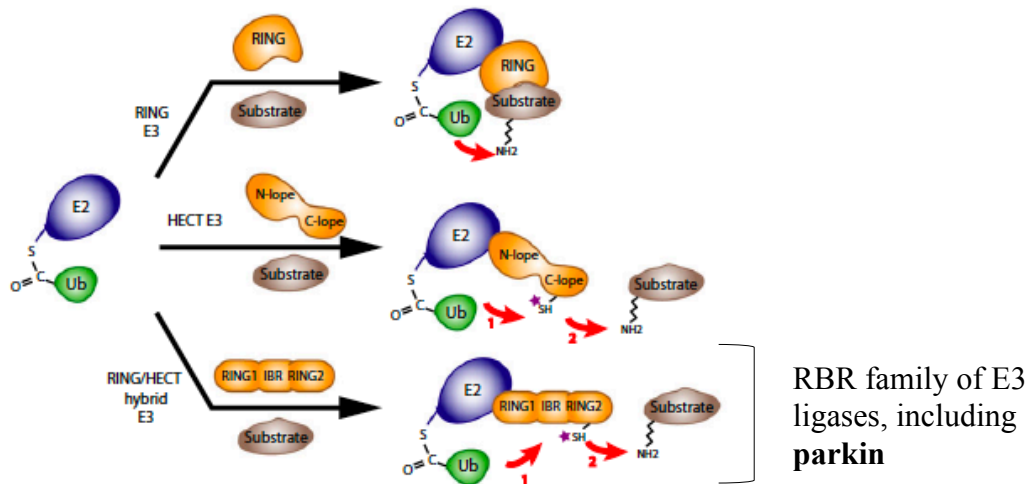


Figure 1.8: The E3-dependent ubiquitination pathway.

a, Schematic representation of the enzyme-dependent cascade of ubiquitination. The process is ATP-dependent and results in monoubiquitin or polyubiquitin chains addition on substrates inducing signaling or degradation pathways. **b**, E3 ubiquitin ligases are classified into three families, distinguished based on their structure and chemistry. Parkin, part of the RBR family of E3 ligases, is classified as a RING/HECT hybrid. Mono, monoubiquitination; poly, polyubiquitin chain addition; NF-κB, nuclear factor κB; and K, lysine. Modified from Seirafi et al., 2015.

1.10.7.2 Autoinhibition and catalytic mechanism

Under basal conditions, soluble parkin is natively found in an autoinhibited state when examined from the perspective of E3 activity. Multiple structural mechanisms control parkin's activity. Firstly, the RING2 domain, site of its catalytic activity, is blocked by RING0, which has been observed in other RBR E3 ligases, including human homolog of Ariadne (HHARI) and heme-oxidized IRP2 ubiquitin ligase-1 (HOIL1)-interacting protein (HOIP), the catalytic subunit of the linear ubiquitination chain assembly complex (LUBAC). Similar to parkin, the Ariadne domain of HHARI and the ubiquitin associated (UBA) domain of HOIP both occlude their catalytic sites²⁴⁶⁻²⁴⁸. Studies have shown that an N-terminal deletion of parkin, including its RING0, results in an increase in its E3 ligase function²³¹⁻²³³. Secondly, parkin's RING1 domain, the site of E2 binding, is obstructed by the protein's Ubl domain and REP linker. Deletions of both regions increases the protein's binding to the E2 enzyme and consequently increases its E3 activity^{225,232}. Thirdly, a 50Å distance between the E2 site and parkin's catalytic cysteine (C431) prevents the transfer of ubiquitin.

The catalytic mechanism of parkin requires two steps: one, the C-terminus of ubiquitin covalently binding to parkin's catalytic cysteine through a thioester intermediate; and two, and acyl-dependent transfer of ubiquitin to an amino group of a substrate. For these steps to take place, the parkin protein requires activation, which will be covered in section 1.10.8.1. Of note, the RING2 catalytic cysteine is conserved among E3 ligases of the RBR family. Furthermore, it has been suggested that two conserved amino acids follow the catalytic cysteine in parkin in a linear arrangement, a histidine and an acidic

residue, forming a catalytic triad. The histidine is proposed to act as a general base to promote catalysis²³¹⁻²³³.

1.10.7.3. Parkin substrates

Parkin ubiquitinates a plethora of proteins through multiple types of ubiquitin chains, including K48, K63, K11 and K6²⁴⁹. These include cytosolic and outer-mitochondrial membrane proteins leading to regulatory or degradation pathways depending on the type of ubiquitination^{250,251}. Of note, it has been suggested that parkin interacts with other PD proteins, including α -synuclein, through direct binding, ubiquitination or functional interaction, which could suggest some converging pathways in PD pathogenesis²⁵². Parkin can also act as its own substrate through K6-mediate autoubiquitination, suggesting auto-regulation of its cellular levels and activity²⁵³. A literature review identified selected parkin-associated substrates, their physiological functions, the type of ubiquitination, and whether parkin-mediated ubiquitination induces degradation, as summarized in the following table (**Table 1.3**).

Substrate	Physiological function	Ubiquitination	Degradation
Ataxin-2	Polyglutamine	Poly	Yes
Ataxin-3	Polyglutamine	Poly	Yes
Bcl-2	Anti-apoptotic protein	Mono	No
CDCrel-1	Synaptic vesicle associated GTPase	Poly	Yes
CDCrel-2a	Synaptic vesicle associated GTPase	Poly	Yes
Cyclin E	Cell cycle regulating protein	Poly	Yes
Drp1	Mitochondrial fission protein	Poly	Yes
Eps15	Internalizes EGF-R	Mono	No
F-Box - Fbw7 β	Multisubunit ubiquitin ligase	Poly	yes
FBP1	Regulates c-myc mRNA	Unknown	Yes
Hsp70	Molecular chaperone	Mono	No
I κ ky	Component in NF κ B signaling	Poly	No
Lim Kinase 1	Phosphorylates cofilin	Poly	Yes
Miro	Mitochondrial anchor protein	Unknown	Yes
Mitofusin-1/-2	Mitochondrial fusion protein	Poly	Yes
p38	Aminoacyl t-RNA synthetase cofactor	Poly	Yes
Pael-R	G-protein coupled receptor	Poly	Yes
PARIS	Transcriptional repressor of PGC-1 α expression	Poly	Yes
PDCD2-1	Involved in apoptosis, inflammation and proliferation	Poly	Yes
PICK1	Synaptic scaffolding protein	Mono	No
PLC γ 1	Hydrolyzes lipids	Unknown	Yes
RanBP2	Interact with nuclear pore complex	Poly	Yes
SynaptotagminXI	Membrane trafficking protein	Poly	Yes
Synphilin-1	Interact with α -synuclein	Poly	No
TRAF2	Component in NF κ B signaling	Unknown	No
VDAC	Mitochondrial ion channel	Poly	No
α -Sp22	Lewy body component	Poly	yes
α / β -tubulin	Cytoskeletal components	Unknown	Yes
β -catenin	Component in Wnt signaling	Unknown	Yes

Table 1.3: Selected parkin's putative substrates.

Selected list of parkin's substrates, identified *in vitro* and *in vivo*, including their physiological functions, form of ubiquitination, and the resulting degradation or absence thereof . Mono, monoubiquitination; and poly, polyubiquitin chain addition.

1.10.7.4 EOPD-linked mutations

Parkin's PD-linked mutations induce several effects, including compromising the protein structural integrity, interfering with substrate binding, and affecting its enzymatic activity. For example, mutations C212Y, C289G and C441R alter the zinc coordination capability of the RING domains, whereas R42P, K211N and T351P affect the protein's stability and its folding. C431F and G430D disrupt parkin's E3 activity and T240R prevents E2 binding. Other mutations such as R33Q, D280N, G328E and T415N, while they don't affect the protein's enzymatic activity, their effects are less understood. A detailed list of parkin's PD-linked mutations and their effects is in the appendix section (**Appendix III**)²³³. However, a recent report discusses at great length the link between parkin PD-linked mutations, their function, structure and and pathogenicity²⁵⁴. For example, the significance of the G328E mutation in the pathogenesis of EOPD is put in question, as it has only been reported in a compound heterozygous case. Additionally, previous mutations with less understood effects have been further explored. The R33Q mutation results in a destabilized Ubl domain, and the T415N mutation affects parkin's ligase activity and involvement in mitophagy²⁵⁴. Furthermore, Yi et al. have developed a framework for classifying the pathogenicity of parkin mutations based on the integration of clinical and functional evidence. Out of 51 naturally occurring parkin mutations, 13 have been deemed pathogenic, while the rest were classified as benign or with uncertain significance²⁵⁴.

1.10.7.5 Parkin's post-translational modifications

Furthermore, several published studies point to post-translation modifications (PTM)

altering parkin's structure and function. Nitrosative stress, including S-nitrosylation of parkin at C323 for example, inhibits its E3 activity²⁵⁵⁻²⁵⁸. It is believed that this modification induces an initial increase in the protein's E3 activity, followed by inactivation. More sites of S-nitrosylation have been identified in the RING1, IBR and RING2 domains²⁵⁹. Dopamine metabolism, as mentioned earlier, produces toxic dopamine quinones able to form adducts onto parkin, mapped to C268 and C231 of the RING1 domain, and inactivate the protein^{260,261}. This modification may be the most physiologically relevant given the specific DA neurons-localized pathology of parkin-linked EOPD. Additionally, it was reported that MPTP or H₂O₂-induced stress results in parkin sulfonation (oxidation) at specific cysteine residues, including C211, C253, C268, C289, C431, and C441, which results in alterations in the protein's solubility and reported E3 function. The reports suggested an initial increase in parkin's E3 activity, followed by inhibition²⁶². We replicated those results using increasing concentration of H₂O₂ (unpublished data – **Appendix IV**). Additional PTMs on parkin are sumoylation²⁶³, neddylation²⁶⁴ and truncation²⁶⁵, of which the former two are suggested to positively regulate the protein's E3 ligase activity. Published parkin PTMs, including phosphorylation at Ser65, and sulphydration at C95, a residue that is human specific and relevant to our work, are depicted in the following graph²⁶⁶ (**Figure 1.9**). Ser65 PINK1-dependent phosphorylation of parkin activates the protein and induces its mitochondrial translocation, which will be discussed in more details in the next section.

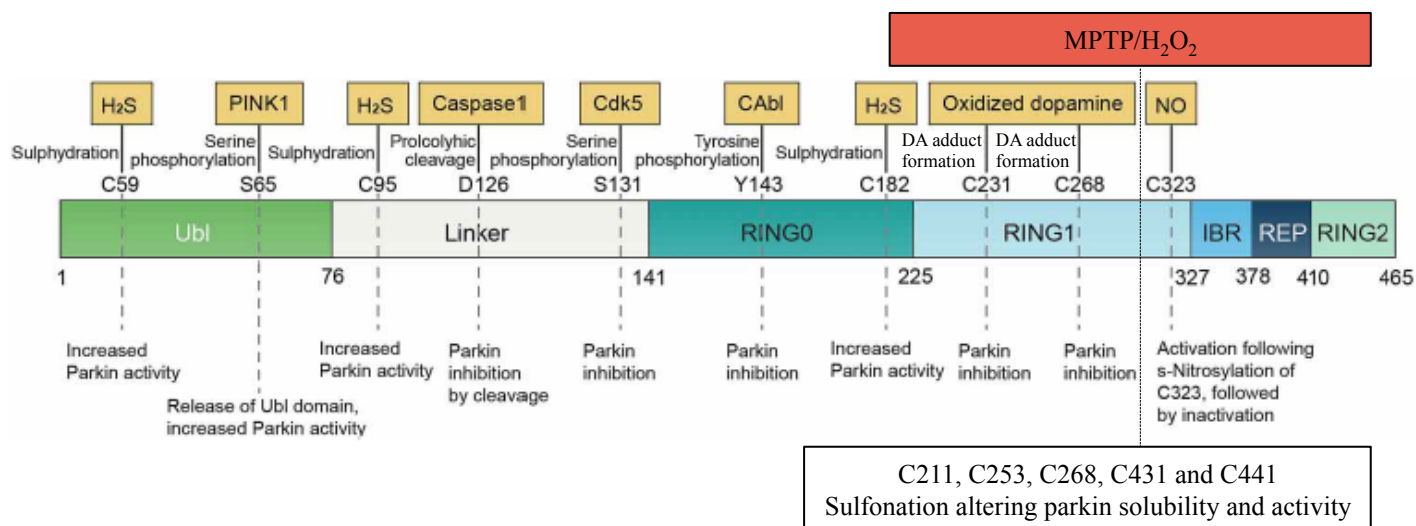


Figure 1.9: Post-translational modification affecting parkin's structure and function.

Selected post-translational modification (PTMs) altering parkin's activity, identified from *in vitro* and *in vivo*, including but not limited to oxidation-mediated cysteine modification such as through dopamine adduct formation, S-nitrosylation and sulfonation; and PINK1-mediated serine phosphorylation such as at Ser65. DA, dopamine. Modified from Panicker et al., 2017.

An accumulation of parkin-mediated polyubiquitin chains on outer-mitochondrial membrane proteins, and through an interplay with PINK1, mitochondrial degradation, also known as mitophagy is induced, which is discussed in the next section.

1.10.8 PINK1-parkin-mediated mitophagy

1.10.8.1 Parkin activation

Initial studies focused on the Ubl domain as the target region of parkin activation. Structural studies had identified an I44-dependent interaction between the Ubl and RING1 domains, obstructing the E2 binding site. Further studies, identified several Ubl binding partners, including the SH3 domain and UIMs of endophilin A1, ataxin-3 and proteasomal subunits²²⁸⁻²³⁰, through the same hydrophobic surface centered around I44, dissociating the Ubl domain from RING1 and allowing for E2 binding.

However, in recent years, a PINK1-dependent phosphorylation of parkin at Ser65 (a residue in the Ubl domain), has gained strong ground as a major contributor to parkin activation and parkin recruitment to depolarized mitochondria²⁶⁷⁻²⁶⁹. However, when analyzing the non-phosphorylatable S65A mutant, it was apparent that its activation and recruitment were not completely impaired, and that a secondary PINK1 substrate involved in parkin activation was needed. This led to the breakthrough discovery that ubiquitin itself is also PINK1-phosphorylated at Ser65^{249,270}. Phosphorylated ubiquitin directly activates parkin and enhances the rate of E2-ubiquitin discharge. Furthermore, a high affinity of parkin-phospho-ubiquitin binding was observed, which was increased 20-fold when parkin itself was phosphorylated by PINK1²⁷¹⁻²⁷³; expression of the non-

phosphorylatable S65A ubiquitin in cells decreased parkin activation and recruitment to mitochondria; and mutating both parkin and ubiquitin at Ser65 inhibited parkin's activation²⁷³.

1.10.8.2 Parkin recruitment

PINK1 accumulates on the outer membrane of depolarized and damaged mitochondria inducing parkin and ubiquitin phosphorylation and activation, and parkin recruitment. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) has been the most common treatment used in cellular paradigms to cause mitochondrial deficits, leading to parkin recruitment, ubiquitination of outer-mitochondrial membrane proteins, and induction of mitophagy²⁶⁷⁻²⁶⁹. These results suggest that parkin and PINK1 are involved in a common pathway regulating mitochondrial quality control and the clearing of selective damaged mitochondria²⁷⁴. The model proposes that under normal conditions, parkin is inactive in the cytoplasm, under autoinhibition, and PINK1 is imported to the mitochondria outer membrane via the (translocase of outer mitochondrial membrane) TOM40 and TOM20 complexes, and further cleaved by mitochondrial proteases (mitochondrial processing peptidase, MPP; and presenilins-associated rhomboid-like protein, PARL)²⁷⁵⁻²⁷⁷. To maintain PINK1 levels under normal healthy conditions, the cleaved protein is degraded rapidly in the cytosol through the N-end rule pathway²⁷⁸. However, damaged mitochondria with defects in electrochemical gradient accumulate PINK1 on their outer membrane, with its kinase domain on the cytosolic side^{279,280}. This stabilization of PINK1 induces the cascade of parkin and ubiquitin activation, parkin recruitment, polyubiquitination of several outer mitochondrial membrane proteins and the autophagic

clearing of the organelles^{267,268}.

The following graph depicts the PINK1-dependent parkin activation and recruitment resulting in mitophagy²²⁴ (**Figure 1.10**).

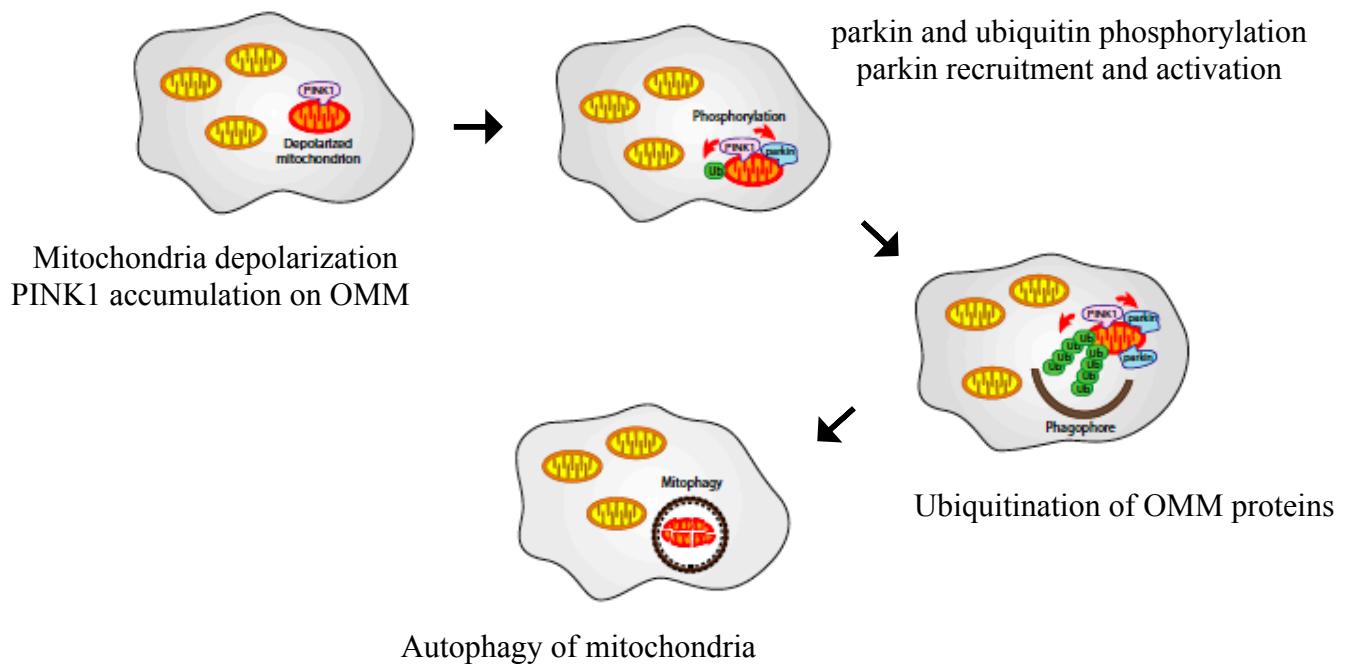


Figure 1.10: PINK1-dependent parkin activation and mitophagy.

Graphical depiction of the mitophagy pathway, including PINK1-mediated phosphorylation of parkin and ubiquitin, parkin recruitment to depolarized mitochondria, and the degradation of mitochondria through autophagy. OMM, outer mitochondrial membrane. Modified from Seirafi et al., 2015.

1.10.9 Other reported parkin functions

Complementary to its E3 ubiquitin ligase activity and role in mitophagy, parkin's ubiquitination of outer mitochondrial proteins such as optic atrophy 1 (OPA1), mitochondrial Rho GTPase (Miro) and mitofusin 1 and 2 (MFN1/2) is implicated in the balance of fission and fusion and mitochondrial motility. It is believed that this process allows for the proper isolation of dysfunctional mitochondria from the mitochondrial network for mitophagy to ensue²⁸¹. Part of its role in mitochondria quality control, studies have shown that parkin in collaboration with PINK1 repairs damaged mitochondria in response to oxidative stress, by packaging them in mitochondrial derived vesicles (MDV). These MDVs, containing oxidized proteins, carry the damage cargo for lysosomal degradation^{282,283}. Additionally, reports have observed parkin's function in the selective turnover of new mitochondria²⁸⁴, and increasing mitochondrial transcription through transcription factor A, mitochondrial (TFAM)²⁸⁵.

Furthermore, parkin has been observed to promote cell survival by activating pro-survival pathways by decreasing activation of c-jun N-terminal kinase^{286,287}, increasing nuclear factor κ B (NF- κ B) to maintain mitochondrial integrity²⁸⁸, and exerting pro-mitochondrial effects, where its expression is inversely correlated with cytochrome c release and the pro-apoptotic pathway²⁸⁹.

Alternatively, parkin has been suggested to act as a tumour suppressor, and its transcription regulated by p53²⁹⁰. It has been reported that parkin mediates the Warburg effect of P53 on glucose metabolism²⁹⁰, its inactivation has been observed in several

human cancers, its expression decreased in 30% of human tumours, and parkin-deficient mice presenting with an increase susceptibility to tumourigenesis²⁹¹⁻²⁹³. Additionally, a role for parkin in host defenses against intracellular bacterial pathogens such as *Mycobacterium leprae*²⁹⁴, *Mycobacterium tuberculosis*, and *Salmonella enterica*²⁹⁵ through autophagy-dependent mechanisms, has been reported.

Recent studies have indicated a function for Parkin in the regulation of mitochondrial antigen processing and pathways of inflammation signalling. Antigen presentation is a crucial process in establishing immune tolerance and in immune response against infections and certain cancers. Most studies report an autophagy-mediated antigen presentation, however recently mitochondrial antigen presentation (MitAP) relying on the generation and trafficking of MDVs has emerged as an alternative pathway. Matheoud et al. have reported that inflammation-elicited immune response in the form of MitAP is inhibited by parkin and PINK1, both *in vitro* and *in vivo*²⁹⁶. Furthermore, parkin has been reported to modulate nod-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome activation²⁹⁷, and mitigate stimulator of interferon genes (STING)-induced inflammation²⁹⁸.

1.10.10 Parkin animal models

Most parkin mouse models were generated through full deletion of exons 2, 3, or 7^{94,299-302}. Mice with exons 2 or 3 deletions showed no overt parkinsonian phenotype: no DA neurons loss was observed, and little to no behavioural deficits were detected. Furthermore, mice with parkin exon 3 deletion displayed an increase in striatal glutamate

levels, abnormal dopamine metabolism, alteration in mitochondrial redox proteins and enzymes expression levels, and signs of oxidative stress^{94,95,300}. The latter two will be further discussed in section 1.10.12. Additionally, parkin mice with exon 7 deletion presented with loss of LC neurons and a diminished norepinephrine-dependent startle response³⁰². No aberrant accumulation of parkin's putative E3 ligase substrates has been observed in parkin-deficient mouse brains. However, some reports indicate the accumulation of aminoacyl tRNA Synthetase Complex Interacting Multifunctional Protein 2 (AIMP2), far upstream element-binding protein 1 (FBP1) and parkin interacting substrate (PARIS) in the ventral midbrain of parkin-null mice³⁰³⁻³⁰⁵. In toxin-based models, namely MPTP, parkin-deficient mice showed no further increase in cell loss when compared to WT littermates. Nevertheless, parkin overexpression provided protection against MPTP-induced damage^{301,306,307}. Lastly, bacterial artificial chromosome (BAC) transgenic mouse expressing a C-terminal truncated variant of human parkin (Q311X) in DA neurons exhibited cell loss and reduction in DA availability at the striatum³⁰⁸. Overall, parkin-deficient mice are not considered a very robust model of parkinsonism³⁰¹.

Similar to mice, other parkin-null animal models generated no strong PD-like phenotypes. Parkin deficient rats showed no age-dependent DA cell loss, LB pathology or behavioural deficits. However, human parkin overexpression in rats, protected against 6-OHDA-induced SNc neurodegeneration³⁰⁹. *Drosophila* models of parkin deficiency, presented with a reduced lifespan, cell loss of indirect flight muscles and male sterility, which were attributed to mitochondrial dysfunctions³¹⁰; and zebrafish morpholino

knockdown of parkin generated mitochondrial complex I impairments and DA cell loss in the absence of motor abnormalities³¹¹ The following table adapted from Jagmag et al.¹⁸⁹ summarizes the major parkin-related animal models (**Table 1.4**).

		Promoter	SN neuron loss	Inclusion bodies	Motor impairment
<i>Mus musculus</i> (Mouse)	Exon 3 deletion	–	–	–	–
	Exon 3 deletion	–	–	–	+
	Exon 2 deletion	–	–	–	–
	Exon 7 deletion	–	–	–	–
	Exon 3 deletion	–	ND	ND	ND
	Truncated, Q311X	<i>Slc6a3</i>	+ (DA loss)	–	+
	WT	<i>nse</i>	–	ND	ND
<i>Rattus norvegicus</i> (Rat)	WT	PGK	–	ND	ND
	WT (KO -Exon 4)	–	–	–	–
<i>Drosophila melanogaster</i> (Fruit fly)	p25 insertion (null)	–	–	ND	+
	P21 insertion (null)	–	–	–	+
	Q311X/T240R	<i>ddc-GAL4</i>	+	–	+

Table 1.4: Genomic *prkn* deficient animal models.

Summary table of the most studied parkin-related animal models, including *Mus musculus* (mice), *Rattus norvegicus* (rats) and *Drosophila melanogaster* (fruit flies). The table displays the phenotypes of each model, including, motor impairment, nigral cell loss and LB pathology. WT, wild-type; *Slc6a3*, solute carrier family 6a3; *nse*, neuron specific enolase; PGK, phosphoglycerate kinase; *ddc*, dopa decarboxylase; and ND, no data. Modified from Jagmag et al. 2016.

1.10.11 Parkin's neuroprotective effects

1.10.11.1 Parkin, a neuroprotective agent

Many reports have identified parkin as a multipurpose protective agent³¹². Under several stress paradigms, parkin overexpression was deemed protective and rescued stress-induced phenotypes. In parkin-overexpressed PC-12 cells exposed to ceramide, mitochondrial swelling followed by cytochrome c release and caspase-3 activation, observed in ceramide-mediated cell death, were inhibited³¹³. In post-mitotic primary neurons, including midbrain DA neurons, treated with kainite, a glutamatergic excitotoxin, parkin overexpression attenuated the accumulation of cyclin E and protected against apoptosis³¹⁴. In dopaminergic SH-SY5Y cells manganese treatment induced an endoplasmic reticulum-mediated cell death in a dose-dependent manner, and interestingly increased the expression of parkin with no observable changes in proteasomal activities. Furthermore, overexpressing of parkin in manganese-treated or manganese and dopamine-treated SH-SY5Y cells resulted in inhibition of cell death³¹⁵. Additionally, in SH-SY5Y cells parkin overexpression protected against DA or 6-OHDA-induced cell death, and significantly decreased the levels of ROS and protein carbonyls³¹⁶. Importantly, α -synuclein-induced toxicity in several models, including rats, *drosophila* and cellular paradigms, was rescued by parkin overexpression^{128,317,318}. To date, the mechanisms of parkin's protective effects against a variety of stressors and toxic insults are still not known. Its E3 activity does not fully explain those effects, as using PD-linked parkin mutants with intact or reduced ligase activity had no rescue capacity when compared to WT parkin³¹⁶. Nevertheless, in section 1.10.12, as well as in Chapters 2 and 3, we offer a potential explanation by establishing a novel redox function for parkin, independent but

not mutually exclusive of its E3 ligase activity and involvement in mitophagy.

1.10.11.2 Parkin, a therapeutic target

Given the current understanding of parkin's function as an E3 ubiquitin ligase, and its interplay with PINK1 in regulating mitochondrial health, several therapeutics approaches have been underway. The focus of the therapeutic research for parkin-linked EOPD has been on increasing its basal activity. Interventions that would increase the protein's activation, such as small molecules that mimic phospho-ubiquitin, or decrease its autoinhibitory mechanisms, are believed to enhance parkin's neuroprotective effects. For instance, mutating the Trp403 or Phe463 residues on parkin increases its recruitment to mitochondria²³². Additionally, these parkin-activating mutations rescue S65A parkin³¹⁹ and several pathogenic PD-linked parkin mutations²⁵⁴. Designing small molecules to bind tightly to the pocket occupied by the amino acid side chains, in order to keep the protein in activated state, is another approach. Research has also focused on deubiquitinating enzymes (DUB) as a target. For instance, (ubiquitin specific peptidase) USP30 and USP15 have been reported to inhibit the parkin-PINK1 mitophagy pathway, hence studies are looking into inhibitors of both DUBs^{320,321}. On the other hand, USP8 is suggested as a candidate for agonist design, as it promotes the mitochondrial clearance²⁵³. Overall, parkin-directed therapies have been centered on functions and mechanistic pathways of the protein that have been the focus of most studies since its discovery over 20 years ago.

1.10.12 Research proposal

1.10.12.1 Parkin, a heretofore unrecognized redox molecule?

Parkin's E3 ligase activity and its involvement in mitophagy have been the focus of most reports in the literature over the past two decades^{150,271,322,323}. However, the field has missed several clues potentially pointing to an essential function of parkin in midbrain DA neurons that is independent of its E3 enzymatic activity and the proposed role in mitochondrial quality control.

E3 ligases are generally highly specific to one or possibly a small number of substrates, however parkin presents with a wide array of putative substrates, some of which are listed in **Table 1.3**. Additionally, although some of these substrates' physiological functions and roles in EOPD have been the focus of limited studies, no widely agreed upon and easily reproduced substrate has been identified that upon accumulation would confer neuronal loss *in vivo*; and parkin knockout mouse models have not displayed a robust upregulation of aberrant proteins^{94,95}; with the possible exception of AIMP2, FBP1, and PARIS which have been reported to accumulate in *in vivo* models³⁰³⁻³⁰⁵. Furthermore, several PD-linked mutations in the parkin protein do not affect its enzymatic activity (**Appendix III**). Alternatively, what parkin-deficient mouse models have reported is an alteration in the abundance and expression of 92 proteins, of which 55 are enzymes and 72 are redox-sensitive^{94,95} listed in the table found in the appendix section (**Appendix V**).

When it comes to mitophagy, cell-based paradigms display elegantly the recruitment of parkin to mitochondria after 1 hour of CCCP treatment. However, concerns regarding the overexpression of parkin in cells and the physiological relevance of the strong depolarization of the entire mitochondrial network induced by CCCP have come into question. Furthermore, recent reports have demonstrated PINK1 mediated mitophagy in the absence of parkin³²⁴, while others observed basal mitophagy independent of both PINK1 and parkin³²⁵⁻³²⁷. In experiments with primary neurons, CCCP-mediated parkin recruitment is much more dampened and requires longer CCCP treatments in the presence of lysosomal or apoptosis inhibitors^{328,329}. However, induction of mitophagy has been reported in neurons expressing endogenous levels of parkin using antimycin A/oligomycin A³³⁰. Additionally, electron microscopic analysis in parkin-deficient mice revealed no gross abnormalities in striatal mitochondria⁹⁴. Similar results were observed in an aged mouse deficient of three ARPD-linked gene, *prkn*; *DJ-1*; and *PINK1*³³¹.

As early as 2003, Pawlyk et al. published a study on parkin in human brains, where they reported a shift in parkin's signal into an insoluble SDS-extracted fraction in 3 older brains, that was not observed in 3 young cortices³³². Further studies have observed cysteine-dependent formation of higher molecular weight (HMW) parkin species and shift in solubility in response to oxidative stress driven by H₂O₂ or dopamine under non-reducing conditions^{260,333}. Taken the two aforementioned observations into consideration, it is noteworthy that most of the field have studied parkin's E3 ligase function and role in mitophagy under reducing conditions and in the soluble fraction, and attributed any structural changes to the protein, including insolubility, to a loss of function. In addition,

since 2002 the field has lacked specific, renewable antibodies to detect parkin in human brain³³⁴, as well as the ability to differentiate between reduced species and oxidized HMW and insoluble species of the protein.

1.10.12.2 Hypothesis and aims

Parkin's biochemical and electrophoretic changes in response to oxidative stress prompted us to delve into its primary structure. As mentioned in section 1.10.6, parkin chelates two zinc ions through seven cysteines and one histidine residues in each of its four RING domains. This confers to the protein a large number of cysteine residues, 35 to be exact. When compared to a number of other human proteins, listed in the following table (**Table 1.5**), including other E3 ligases, parkin had the second highest cysteine-to-amino acid ratio with 7.5%, following the sensitive-to-apoptosis gene (SAG) protein with 10.6%. The SAG protein is known to have E3 ligase activity that undergoes redox reactions and can act as a cellular antioxidant^{335,336}.

Cysteine content of human proteins				
Protein name	Gene name	# of amino acids	# of cysteines	Ratio
Alpha-Synuclein	<i>SNCA</i>	140	0	0.0%
c-Cbl	<i>Cbl</i>	472	13	2.8%
Chip	<i>STUB1</i>	303	8	2.6%
DJ-1	<i>PARK7</i>	189	3	1.6%
Dorfin	<i>RNF19A</i>	838	35	4.2%
E6-AP	<i>UBE3A</i>	852	16	1.9%
HHARI	<i>ARIH1</i>	557	32	5.7%
HOIL1	<i>RBCK1</i>	510	32	6.3%
LRRK2	<i>PARK8</i>	2527	64	2.5%
Parkin	<i>PARK2</i>	465	35	7.5%
PINK1	<i>PINK1</i>	581	16	2.8%
SAG - sensitive to apoptosis gene	<i>RNF7</i>	113	12	10.6%

Table 1.5: Cysteine-to-amino acid ratio of selected human proteins.

The cysteine to total amino acid ratio of selected human proteins is displayed. Highlighted is parkin with a ratio of 7.5%, second only to the SAG protein, an E3 ubiquitin ligase with antioxidant and redox functions, with a ratio of 10.6%.

Thiol groups of cysteine residues are known to be highly reactive and involved in maintaining an equilibrium between reducing and oxidizing forces that is essential for normal cell and organ functioning and health, and overall redox balance³³⁷⁻³⁴⁰. Furthermore, thiols can act as sensors of oxidative stress, and are able to react directly with ROS, reactive nitrogen species (RNS) such as nitric oxide (NO), and reactive electrophilic species (RES) such as dopamine quinones. These oxidation-driven reactions induce PTMs at the cysteine residue level, graphically represented in **Extended Data Figure 2.3** (Chapter 2), resulting in structural changes, which can activate or disable the protein^{341,342}. Reinforcing the importance of thiol groups is the abundance of glutathione, a cysteine-dependent antioxidant comprised of three amino acids, in cells and tissues; and its decline implicated in many human disorders including neurodegenerative diseases³⁴³.

As it relates to parkin, the protein undergoes cysteine-dependent biochemical changes in response to ROS, RNS and RES^{258,260,333}; and in parkin-null mouse models alterations in redox enzymes expression levels and upregulation of reduced glutathione are observed, as potential compensatory mechanisms^{76,94,95,300}. Furthermore, while parkin-null mouse models do not present with strong parkinsonian phenotypes, namely DA cell loss and motor deficits, they exhibit signs of oxidative stress, summarized in the following table (**Table 1.6**). Linking it back to parkin-associated EOPD, and the selective vulnerability of DA neurons, parkin-deficient SNc DA neurons exhibit increased basal oxidative phosphorylation and reduced survival, when compared to PINK1 or DJ-1 null DA neurons. In addition, overexpression of WT parkin rescues this phenotype³⁴⁴.

Published model	Summarized main results
Itier et al., 2003	Motor and cognitive deficits Inhibition of amphetamine-induced dopamine release Inhibition of glutamate neurotransmission Increased Dopamine in limbic brain areas Increased Dopamine metabolism by MAO No nigrostriatal dopamine neuron degeneration Decreased levels of dopamine transporter and increased levels of GSH
Goldberg et al., 2003	Normal brain morphology Increased extra cellular dopamine in the striatum Reduction in synaptic excitability No changes in the number of dopamine neurons No changes in the levels of CDCrel-1, synphilin-1 and α -synuclein
Palacino et al., 2004	Decreased abundance of proteins involved in mitochondrial function and oxidative stress Reduction in respiratory capacity in striatal mitochondria No gross abnormalities in striatal mitochondria Decreased levels of proteins involved in protection against oxidative stress Increased protein carbonyl levels
Von Coelln et al., 2004	No impairment in the nigrostriatal dopaminergic system Loss of catecholaminergic neurons Reduction in the norepinephrine-dependent startle response
Perez and Palmiter, 2005	No changes in behavior No differences in catecholamines levels
Periquet et al., 2005	Absence of nigral degeneration Upregulation of a number of proteins that may be acting as compensatory mechanisms against ROS and damage to the cell
Sato et al., 2006	Decreased endogenous dopamine release Upregulation of dopamine receptor binding in the striatum Increased dopamine levels in the midbrain
Oyama et al., 2010	Lower release and uptake of dopamine in young mice Enhanced dopamine overflow by repetitive stimulation in older mice Absence of nigral degeneration

Table 1.6: *In vivo* genomic *prkn* mouse models.

Summary table of published parkin mouse models indicating the absence of motor deficits and nigral degeneration, and highlighting in red the increase in markers of oxidative stress.

Taking all of these observations together, we posit that wild-type parkin functions as a redox-sensitive protein, as mediated by its abundance in cysteine residues. Parkin also functions as an anti-oxidant molecule in that through its own oxidation it can mediate redox-reducing effects onto select ROS, RES and RNS radicals. By inference, parkin's absence in EOPD brains results -among other effects- in increased activation of oxidative stress-related pathways that lead to the selective degeneration of midbrain DA neurons.

To test our hypothesis, we set out to:

Aim 1: Explore parkin's novel redox function *in vitro* using human recombinant protein and its metabolism in human brain specimens, and study its interaction with ROS and dopamine radicals as well as its participation in melanin formation.

Aim 2: Validate our observations from human brain studies *in vitro* and *in vivo* using cellular paradigms and select genetic mouse models; and investigate parkin's effects on the thiol network, namely with an emphasis on glutathione metabolism.

Aim 3: Based on our results from aims 1 and 2, develop and characterize a bi-genic mouse model of parkin deficiency that carries haploinsufficiency at the *Sod2* locus to increase the degree of oxidative stress in adult mice, and which was created to explore the presence of early motor features of parkinsonism.

The three aims stated above will be addressed in chapters 2, 3 and 4, respectively.

1.10.12.3 Thesis format

Following the Department of Cellular and Molecular Medicine (Neuroscience Program) at the Faculty of Medicine of the University of Ottawa guidelines, this thesis is written in a paper-based format.

Chapter 1 is a general introduction reviewing the literature on Parkinson disease and discussing, in more depth, parkin-linked early-onset parkinsonism.

Chapter 2 is the first manuscript that tests aim 1 of our hypothesis. It is entitled **Oxidative Modifications of Parkin Underlie its Selective Neuroprotection in Adult Human Brain**. This manuscript has been submitted to *Nature* on February 24th, 2020. A revision for submission to *Acta Neuropathologica* by early July 2020 is currently in preparation. The original version of the manuscript was uploaded onto bioRxiv on February 24th, 2020. BioRxiv 2020.02.19.953034;
doi: <https://doi.org/10.1101/2020.02.19.953034>

Chapter 3 is the second manuscript that tests aim 2 of our hypothesis. It is entitled **Parkinson Disease-Linked Parkin Mediates Redox Reactions That Lower Oxidative Stress In Mammalian Brain**. This manuscript has been submitted to *Nature* on May 4th, 2020. Following the recommendation of the senior editor, the manuscript is now being revised for submission to *Nature Communications* in July 2020. The original version of the manuscript was uploaded onto bioRxiv on April 27th, 2020. BioRxiv 2020.04.26.062380;
doi: <https://doi.org/10.1101/2020.04.26.062380>

Chapter 4 is the third manuscript that addresses aim 3 of our hypothesis. It is entitled **Validation of an anti-oxidant function by parkin in mammalian brain through the characterization of a bi-genic *prkn*^{-/-}//*Sod2*^{+/-} mouse model**. This manuscript is currently in preparation for submission to peer review in August 2020; it too will be uploaded at bioRxiv prior to formal submission.

Chapter 5 is a general discussion of the results presented in the three manuscripts, the relevance of the work to the field of Parkinson disease research, and considerations regarding research directions that can be pursued in the future.

**Chapter 2: Manuscript 1 - Oxidative Modifications of Parkin
Underlie its Selective Neuroprotection in Adult Human Brain**

Statement of author contribution

In this manuscript, we established a novel function of parkin in mediating redox reactions in human brain. We based our finding on parkin's metabolism in >60 human specimens and mapped its oxidative stress-driven post-translational modifications to a select number of cysteine residues. We validated our human brain observations with recombinant protein. *In vitro* parkin directly reduces H₂O₂, resulting in its cysteine-dependent oxidation and insolubility. In its absence, ROS levels rise in mouse and human brains. In addition to its capacity to quench H₂O₂, parkin conjugated dopamine metabolites and augments melanin formation *in vitro*, in a cysteine-dependent manner mapped to the human specific C95. Additionally, in *Substantia nigra*, parkin co-localized with neuromelanin in LAMP3-positive lysosomes.

Dr. Jackie Tokarew established the experimental set-up and layout, in close collaboration with **Daniel El Kodsi**, specifically in regards to optimizing assays (incubation times, concentrations, reagents, buffers etc.), and discussing published literature to ensure sound scientific methodology and experimental design. **Daniel El Kodsi** measured H₂O₂ levels in human brains, including ARPD specimens, in **Figures 2.1i,m; 2.3g**. The recombinant proteins (r-parkin, and PD-liked variants C431F and G329E) analyzed by circular dichroism and dynamic-light-scattering by our collaborator Dr. Gergely Toth, were prepared and isolated by **Daniel El Kodsi** (**Figure 2.2c,d; Extended Data Figure 2.4e,f,g**). Parkin's direct reaction and reduction of H₂O₂ performed by Dr. Jackie Tokarew in **Figure 2.3a-f**, and **Extended Data Figure 2.5** were established in initial experiments by **Daniel El Kodsi** and further validated in Manuscript 2 (Chapter 3).

mRNA analysis of human brain cortices (data not shown) and further analyses of mRNA human data from the Human BRAINCode database were performed by **Daniel El Kodsi** (**Figure 2.1e**). Validating, in mouse brains, that post mortem intervals (PMI) have no effects on parkin's biochemical changes observed in human brains, was established by **Daniel El Kodsi** in collaboration with Nathalie Lengacher (**Extended Data Figure 2.2d**). Some experiments focusing on oxidizing r-parkin with increasing concentrations of H₂O₂ and/or dopamine, analyzed by mass spectrometry at the proteomics core facility to map cysteine residues modifications were conducted by **Daniel El Kodsi**. All mass spectrometry data were analyzed and compiled by Daniel El Kodsi, including from human brains (**Extended data Figure 2.4e**; **Extended Data Table 2.1**; **Supplementary Table 2.2**). Initial production of aminochrome used by Travis Fehr in r-parkin oxidizing experiments to detect dopamine adduct formation by mass spectrometry were performed by **Daniel El Kodsi**. All M17 cell-related experiments, including dopamine-mediated stress, and viability assay were conducted by **Daniel El Kodsi** (**Figure 2.4 a-e**; **Extended Data Figure 2.6a**). Lastly, **Daniel El Kodsi** contributed to major edits of the manuscript and wrote the materials and methods section, as well as referencing.

Oxidative Modifications of Parkin Underlie its Selective Neuroprotection in Adult Human Brain

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Abstract

The mechanisms by which Parkinson disease-linked parkin confers neuroprotection of human dopamine cells remain elusive. We hypothesized that its cysteines mediate multiple anti-oxidant effects in the midbrain. By studying >60 control specimens, we found that in adult human brain -but not in skeletal muscle- parkin is mostly aggregated and insoluble due to oxidative modifications, such as at C253. *In vitro*, parkin's oxidation directly reduces hydrogen peroxide (H_2O_2) to water. In parkin-deficient human brain, H_2O_2 concentrations are elevated. In dopamine toxicity studies, wild-type parkin -but not disease-associated mutants- prevents neural death by lowering H_2O_2 and sequestering radicals within insoluble aggregates. Parkin conjugates dopamine metabolites at the human-specific residue C95 and augments melanin formation *in vitro*. Using epitope-mapped antibodies, we found that in adult *Substantia nigra* neurons parkin localizes to neuromelanin within LAMP-3/CD63-positive lysosomes. We conclude that parkin's own oxidation, previously considered a loss-of-function event, helps explain its neuroprotective effects in adult midbrain: parkin's many thiols participate in H_2O_2 reduction, dopamine radical conjugation and melanin formation.

Introduction

Bi-allelic mutations in the *PRKN* gene, which encodes parkin, lead to young-onset, autosomal-recessive Parkinson disease (ARPD)¹⁹³. Pathological studies of parkin-deficient brains have demonstrated neurodegeneration that appears restricted to the *S. nigra* and *L. coeruleus*, two nuclei synthesizing dopamine (DA).

Parkin, a principally cytosolic protein, has been associated with diverse cellular functions, foremost related to ubiquitin ligase activity and mitochondrial integrity³⁴⁵. To date, these roles have not yet explained its neuroprotective selectivity, and vertebrate models of genomic *prkn* deletion have failed to reproduce DA cell loss. This observation could be due to compensatory mechanisms, a shorter life span, or the uniqueness of human DA metabolism. The latter is exemplified by the generation of neuromelanin after childhood^{57,346}. Nevertheless, *prkn*-null models have revealed changes in high energy-producing cells in flies³⁴⁷ and in murine brain, which indicated elevated oxidative stress^{94,95,300}. We postulated that parkin promotes redox homeostasis *in vivo*.

Redox equilibrium frequently involves cysteine-based chemistry; there, thiols are subject to oxidative modifications by reactive oxygen-, reactive nitrogen- and reactive electrophilic species (ROS, RNS, RES)³⁴¹, some of which are reversible. Proteins irreversibly conjugated by RES, including by DA radicals, are degraded or sequestered within inclusions. It is thought that this process underlies neuromelanin formation⁵³.

Human parkin contains 35 cysteines¹⁹³. Several reports previously demonstrated its sensitivity to ROS and RES in cells^{262,333}. RNS also attack parkin, and NO-/NO₂-modified variants have been described in cells and tissues²⁵⁷. Such oxidative modifications, which reduce parkin's ubiquitin ligase activity *ex vivo*, have been previously interpreted as a 'loss-of-function' *in vivo*^{256,258,260,262}. In contrast, we hypothesized that parkin's oxidation ensures redox homeostasis by actively neutralizing ROS, RNS and DA radicals, and that in parkin's absence their accumulation leads to selective neurodegeneration in *PRKN*-linked ARPD. Hence, we explored parkin's metabolism in adult human midbrain vs. cortex, spinal cord and skeletal muscle, tested its contribution to redox equilibrium, and studied parkin modifications during DA metabolism.

Results

Parkin is mostly insoluble in the ageing human midbrain

Parkin's metabolism in the human brainstem has remained largely unexplored³³². We serially fractionated >40 cortices (ages, 5-85 years) and 20 midbrain specimens (26-82 years) from control individuals (**Fig. 2.1**, **Extended Data Fig. 2.1**; **Supplementary Table 2.1**). We found that before age 20 years, nearly 50% of cortical parkin was found in soluble fractions generated by salt [Tris-NaCl]- and mild detergent [Triton X-100]-containing buffers (**Fig. 2.1a,b**; **Extended Data Fig. 2.1a**). In contrast, after age 50 years, parkin was found almost exclusively (>90%) in the 2% SDS-soluble fraction and 30% SDS extract of the final pellet in both midbrain and forebrain specimens (**Fig. 2.1a-c**). The requirement for SDS to solubilize parkin suggested it had been modified, consistent with its co-distribution with LC3B, a marker of protein aggregation (**Fig. 2.1d**). In soluble fractions from older humans, we did not detect any truncated species of parkin using several, specific antibodies. Despite the protein's solubility loss, *PRKN* mRNA was detectable in individual neurons from all age groups (**Fig. 2.1e**). Transcript levels were independent of age (**Extended Data Fig. 2.1d**).

Using a logistic regression model, we found that parkin's solubility in human brain correlated with age (**Fig. 2.1f**), where the age coefficient was -0.0601 (95% CI: -0.106 to -0.024; P=0.004). When using age to separate Tris-soluble from insoluble parkin, the area under the receiver operating characteristic curve was 0.8. This transition to insolubility occurred between the ages of 28 (low sensitivity; high specificity) and 42 years (high sensitivity; low specificity). The partitioning was not caused by tissue freezing, was

independent upon *post mortem* interval and sex, and it was not affected by the pH during extraction (**Extended Data Fig. 2.2**).

An age-dependent partitioning of parkin was not seen for any other protein examined, such as PD-linked proteins (*e.g.*, DJ-1 and α -synuclein; **Fig. 2.1a,g**) and organelle-associated markers, *e.g.*, cytosolic glyoxalase-1, peroxiredoxin-1 and -3; endoplasmic reticulum-associated calnexin; mitochondrial VDAC and MnSOD (**Fig. 2.1h; Extended Data Fig. 2.1b,c**; and data not shown). However, partitioning was organ- and species-specific. In contrast to human brain, parkin remained soluble in skeletal muscle and spinal cord specimens from older individuals (**Fig. 2.1i; Extended Data Fig. 2.1e**). In the brains of aged mice, rats and cynomolgus monkey, parkin remained soluble even when processing conditions were matched to those of human tissues (**Extended Data Fig. 2.1f**). We concluded that parkin's metabolism is distinct during human brain ageing.

Parkin is oxidized in adult human brain

When brain fractions were examined under non-reducing conditions, we detected parkin proteins ranging in M_r from >52 to 270 kDa, invariably in the form of smears (**Fig. 2.1j**). These species were most abundant in SDS extracts and reduced to parkin's monomer (~52.5 kDa) by adding dithiotreitol (DTT) or β -mercaptoethanol. This is shown, for example, in specimens from both normal and diseased cases (**Fig. 2.1j,k; Extended Data Fig. 2.1g**). No such reactivity was seen in SDS-extracts of *PRKN*-linked ARPD brains, demonstrating specificity. Examples of oxidative modifications are shown in **Extended Data Fig. 2.3**. Under the same conditions, high M_r protein variants were rarely seen for

other PD proteins, *e.g.*, DJ-1 and α -synuclein. Of note, the formation of high M_r parkin was not due to secondary oxidation *in vitro*, because specimens were fractionated in the presence of iodoacetamide (IAA) to protect thiols; they also did not arise from ubiquitin adducts, which are not removable by reducing agents.

These findings demonstrated that a pool of parkin had undergone oxidation *in vivo*. Indeed, using logistic regression analyses, we found that in the same specimens H_2O_2 concentrations correlated with age (**Fig. 2.1l**). Moreover, parkin's solubility change paralleled the rise in H_2O_2 ; the latter's coefficient was -0.939 (95% CI: -2.256 to -0.248; $P=0.0415$; **Fig. 2.1m**).

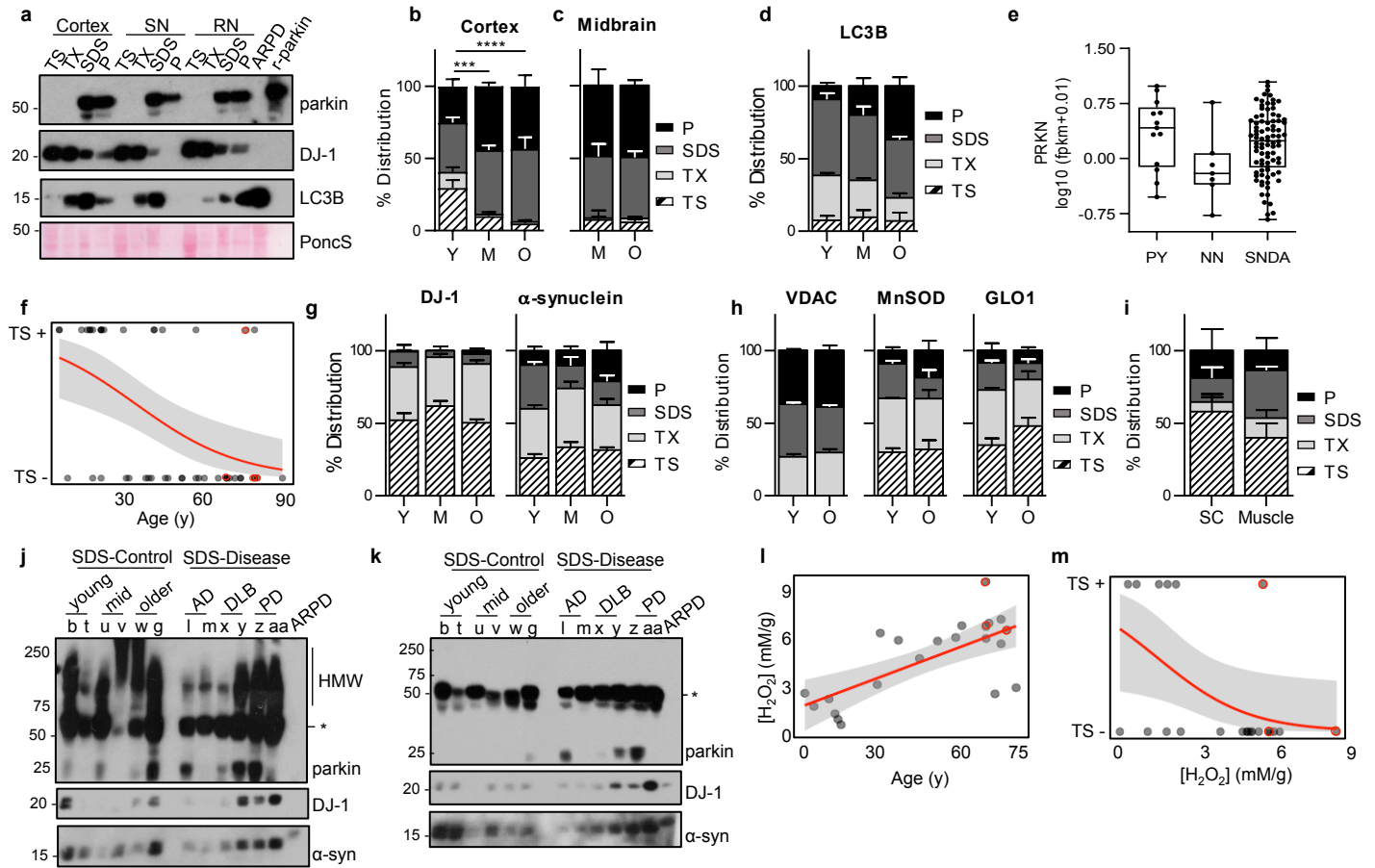


Figure 2.1: Parkin's loss of solubility in adult human brain correlates with age and oxidative stress.

(a) Representative Western blots of parkin, DJ-1, and LC3B distribution in human cortex, *S. nigra* (SN) and red nucleus (RN) serially fractionated into Tris-NaCl buffer-soluble (TS), Triton X-100-soluble (TX), 2% SDS-soluble (SDS) extracts and the pellet (P) lysed in 30% SDS-containing buffer. SDS extracts from *PRKN*-linked Parkinson disease (ARPD) brain and recombinant, human parkin (r-parkin) are included.

(b-c) Relative distribution of parkin signal within each fraction for cortex and midbrain grouped by age ranges: young (Y; ≤ 20 y; $n=13$); mid (M, >20 y, <50 y; $n=15$ cortex, 6 midbrain); older (O, >50 y; $n=13$ cortex, 14 midbrain). Data shown as mean $\pm$ SEM. Significance in protein distribution between soluble (TS+TX) and insoluble (SDS+pellet) fractions was determined using 2-way ANOVA (** $p<0.001$; **** $p<0.0001$). Additional Western blots in **Extended Data Fig. 2.1a-c**.

(d) Signal distribution for LC3B in human cortices as in (b). ($n=3-5$ per age group).

(e) Quantification of log-transformed *PRKN* mRNA signals from individual pyramidal neurons (PY), leukocytes (non-neuronal cells; NN) and *S. nigra* dopamine neurons (SNDA) isolated from healthy controls (ages, 59-89y).

(f) Logistic regression analysis of parkin solubility in cortices as a function of age ($n=45$). Each brain is represented by an individual dot; red dots denote cases of late-onset Parkinson's not linked to *PRKN*; the logistic regression line (in red) and 95% confidence intervals (grey) are shown.

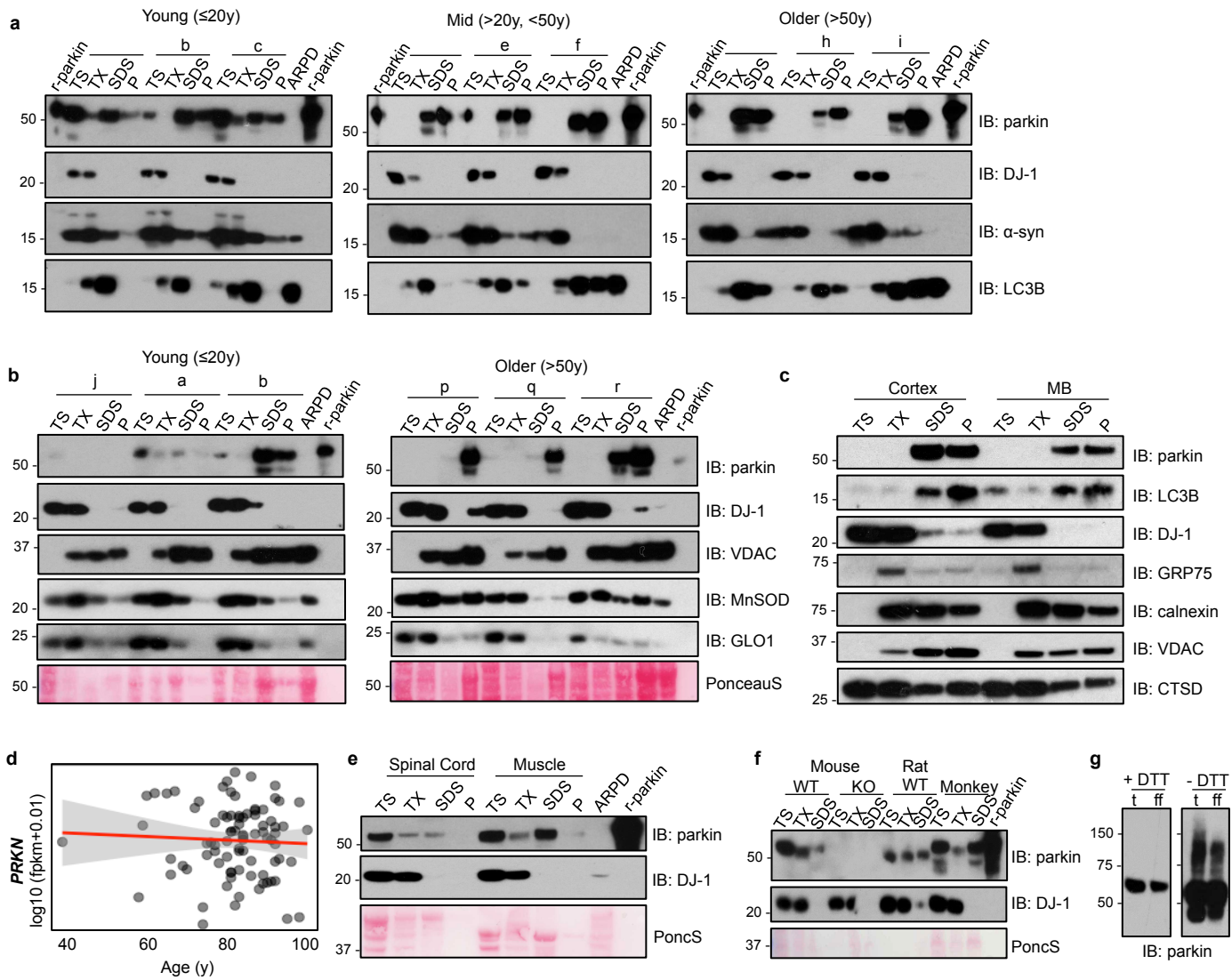
(g-h) Relative distribution of DJ-1, α -synuclein and VDAC, MnSOD and glyoxylase (GLO1) as described in (b).

Representative Western blots in **Extended Data Fig. 2.1b,c**.

(i) Relative distribution of parkin as in (b) for human spinal cord ($n=4$) and skeletal muscle specimens ($n=6$), from donors aged 50-71y. Representative Western blots are in **Extended Data Fig. 2.1e**.

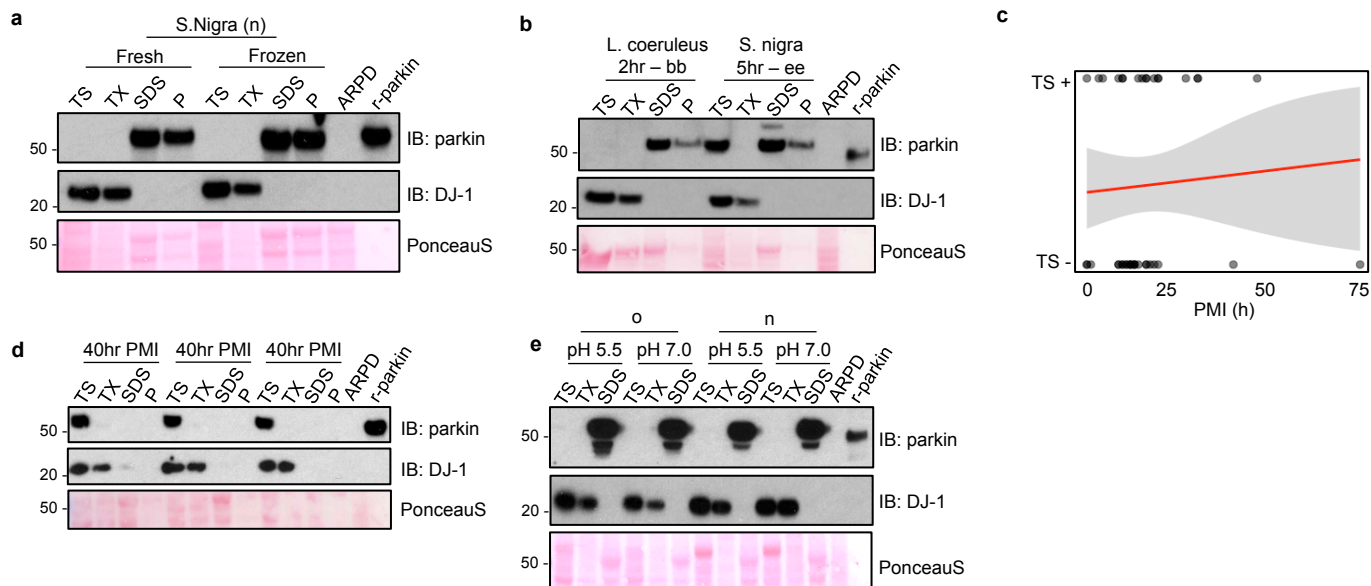
(j-k) Western blots of parkin, DJ-1, and α -synuclein in SDS cortical fractions from normal controls (3 age groups) and 6 patients including Parkinson's (PD), Alzheimer disease (AD) and dementia with Lewy bodies (DLB). The same lysates were separated by SDS-PAGE under (j) non-reducing and (k) reducing conditions.

(l-m) Linear regression analysis of H_2O_2 concentrations in control cortices (mM/g tissue) as a function of age (l), and (m) logistic regression analysis of parkin solubility as a function of H_2O_2 levels in the same specimens ($n=20$) as in (f). Red circles denote three disease cortices (AD; DLB; PD).



Extended Data Figure 2.1: Parkin becomes insoluble in aged brains of humans but not other mammals.

- (a) Western blots of parkin, DJ-1, α -synuclein and LC3B distribution in 9 representative human cortices identified by lowercase letter code (see **Supplementary Information Table 1**). Tissue fractionation and age ranges were as described in Fig. 1; gels were run under reducing conditions. ARPD lysates and r-parkin are included as controls.
- (b) Western blots of parkin, DJ-1, VDAC, MnSOD and glyoxylase-1 proteins, and Ponceau S staining in serially fractionated human cortices from younger ($n=3$) and older ($n=3$) individuals. Quantification of relative protein distribution is shown in **Fig. 2.1h**.
- (c) Western blot of indicated proteins from serially fractionated cortex and midbrain from a single donor as described in (a).
- (d) Linear regression analysis of log transformed *PRKN* transcripts as a function of age in human control *S. nigra* dopamine neurons where each dot represents values for a single neuron as shown in **Fig. 2.1e**.
- (e) Western blot of parkin and DJ-1, and Ponceau S staining of serial fractions from representative human spinal cord and skeletal muscle tissues from individuals $\geq 50y$. Relative protein distribution is shown in **Fig. 2.1i**.
- (f) Western blot of parkin and DJ-1 and Ponceau S staining of serial fractions from whole brains of wild-type (WT; 8 mths of age) and *prkn* knock-out (KO) mice, rat (WT; 14 mths) and cortex from cynomolgus monkey (60 mths).
- (g) Western blots of TXS fractions from 2 human cortices (letter codes t; ff) run under reducing (+DTT) and non-reducing (-DTT) conditions.



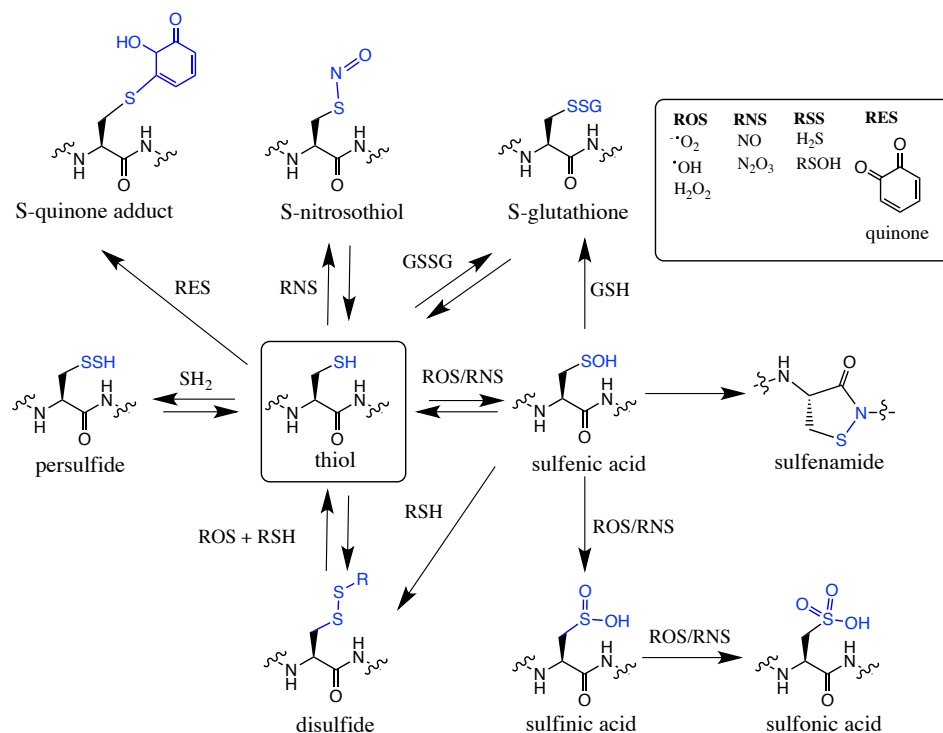
Extended Data Figure 2.2: Parkin solubility is not altered by tissue freezing, length of *post mortem* interval or pH changes.

(a-b) Western blot of parkin and DJ-1 distribution of (a) human *S. nigra* (brain code n) lysates processed in parallel with vs. without a freeze/thaw step prior to serial fractionation, and (b) from *L. coeruleus* and *S. nigra* which were collected within 2-5 hrs after death prior to freezing.

(c) Logistic regression analysis of parkin solubility as a function of length for *post mortem* interval (PMI; in hrs); the logistic regression analysis line (red) and 95% confidence intervals (grey) are shown (n=45 cortices).

(d) Immunoblots for endogenous parkin and dj-1 signals from serially extracted, WT mouse brains (n=3) dissected following a 40 hr *post mortem* interval at 4°C.

(e) Western blots of parkin and DJ-1 distribution and Ponceau S staining for fractions of human cortices serially extracted in parallel using standard buffers with varying pH, as indicated.



Extended Data Figure 2.3: Summary of redox-related thiol chemistry.

Schema of select, reversible and irreversible cysteine modifications that can occur on thiols (-SH) due to attacks by reactive oxygen species (ROS), -nitrogen species (RNS), -sulfur species (RSS) and -electrophilic species (RES), which include quinones. Modified from Alcock *et al.*, 2018.

Redox stressors directly modify parkin and alter its solubility

To investigate how redox stress altered solubility, we exposed purified, recombinant (r-), tag-less, human parkin to oxidants *in vitro*. Exposure of r-parkin to rising concentrations of H₂O₂, DA, the DA metabolite aminochrome, and mitochondrial toxins (*e.g.*, CCCP and rotenone), generated high *M_r* smears and progressive insolubility (**Fig. 2.2a; Extended Data Fig. 2.4a-c**). Some of these changes were reversible, as shown for insoluble, H₂O₂-oxidized r-parkin upon DTT exposure (**Fig. 2.2b**). Others could not be reduced because of irreversible modifications (*e.g.*, **Extended Data Fig. 2.4a-c**). Blocking access to parkin's thiols prevented oxidation (see below). Demonstrating its sensitivity to bi-directional redox changes, r-parkin exposure to excess DTT also rendered it insoluble (**Extended Data Figs. 2.3, 2.4d**).

Oxidative stress alters parkin's structure

The progressive insolubility of parkin under redox stress suggested that the nascent protein had undergone structural change. We tested this by dynamic-light-scattering (DLS) and far-UV-circular dichroism (CD). When we monitored r-parkin by DLS during spontaneous oxidization (**Extended Data Fig. 2.4g**), we observed a gradual shift in hydrodynamic diameter from 5.1 nm at the start, representing a folded monomer, to multiple peaks with larger diameters 5 hrs later. The latter indicated multimer formation, which was reversible by DTT.

When we analyzed the effects of spontaneous oxidation in naïve and H₂O₂-treated r-parkin by CD (**Fig. 2.2c,d; Extended Data Fig. 2,4e,f**), soluble fractions initially

contained both α -helically ordered as well as unstructured proteins (**Fig. 2.2b**; **Extended Data Fig. 2.4e**). Five days later, r-parkin preparations were centrifuged and fractions re-analyzed (**Fig. 2.2d**; **Extended Data Fig. 2.4f**). There, we found a marked shift to increased β -pleated sheet-positive r-parkin in the insoluble fractions. This change was more pronounced in H_2O_2 -oxidized r-parkin, as expected (**Extended Data Fig. 2.4f**).

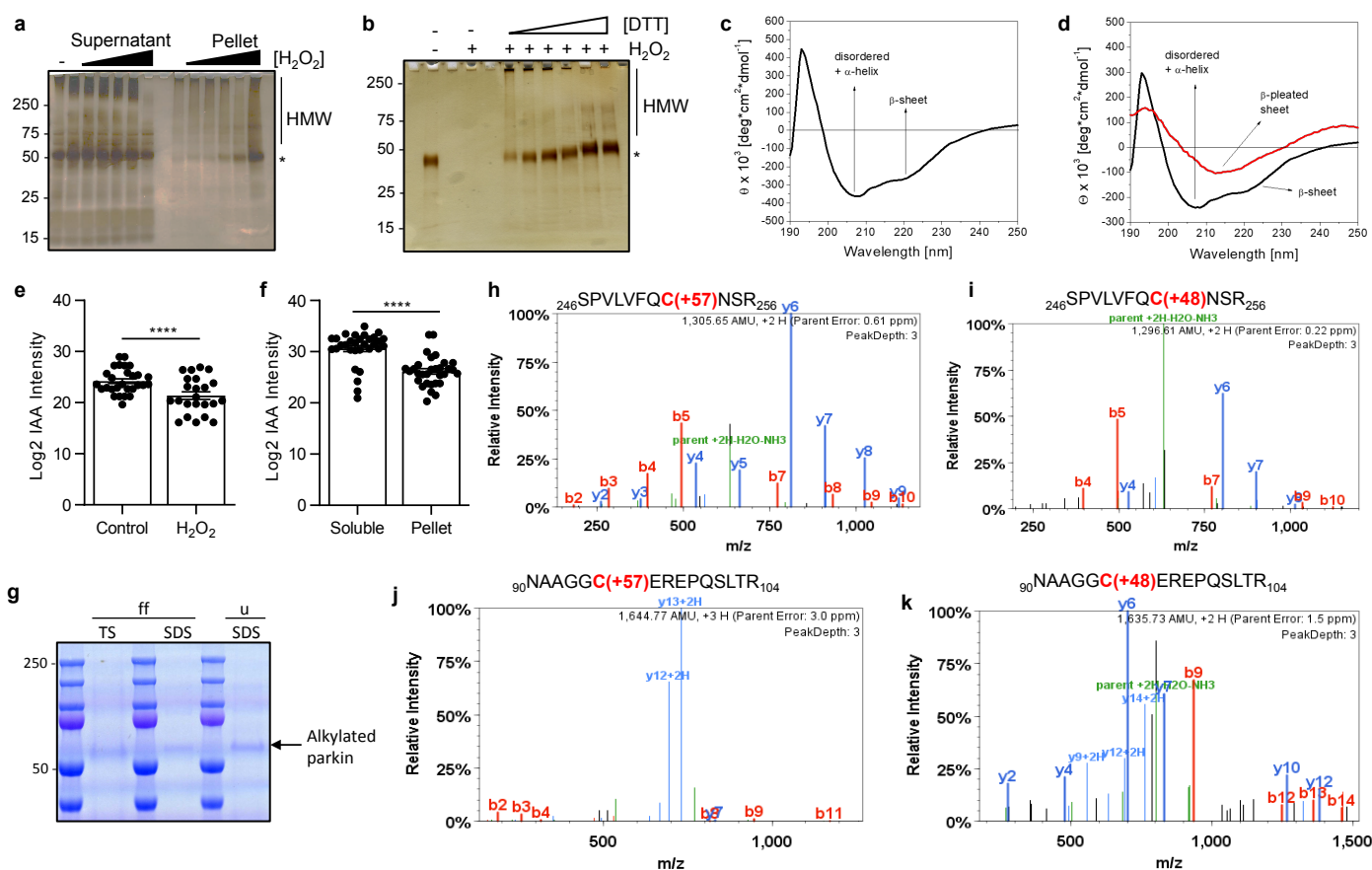
H_2O_2 modifies parkin at multiple cysteines

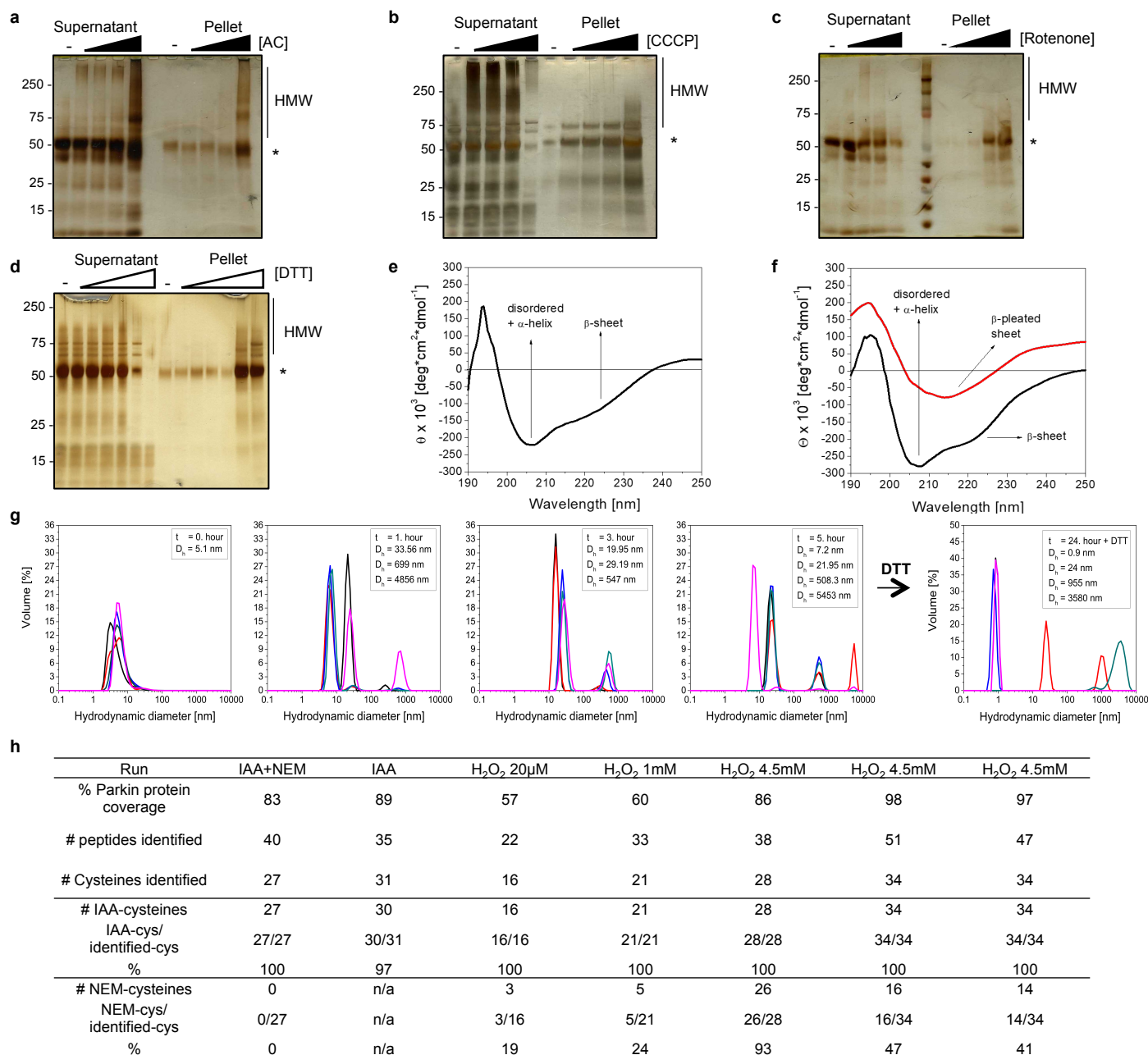
To determine whether oxidation of cysteines caused parkin's insolubility, we employed liquid chromatography-based mass spectrometry (LC-MS/MS). We used a serial IAA-DTT-NEM fingerprinting approach to differentiate reduced thiols (tagged by IAA) from reversibly oxidized thiols (reduced by DTT and tagged by N-ethylmaleimide (NEM)). We first asked whether oxidation led to detectable changes in thiol redox state regardless of solubility. At higher H_2O_2 concentrations, 32 of r-parkin's 35 cysteines (91.4%) were identified; >93.8 % were found modified at least once. Using Scaffold PTM-software, we recorded the anticipated rise in NEM-tagged cysteines following H_2O_2 treatment (**Extended Data Fig. 2.4h**). When quantifying thiol modifications by MaxQuant software³⁴⁸, we found a significant loss of IAA signals for r-parkin's cysteines in H_2O_2 -treated samples ($P=0.0016$; **Fig. 2.2e**), which reflected a loss in reduced thiols.

When comparing cysteine oxidation in soluble vs. insoluble fractions of untreated and oxidized r-parkin, IAA signals were significantly decreased in the pellets ($P<0.0001$; **Fig. 2.2f**). Modifications at methionines did not correlate with r-parkin insolubility. In these studies, residue C253 (RING1 domain) was frequently detected as oxidized. These results

established that H₂O₂-induced oxidation of cysteine-based thiols confers r-parkin insolubility.

We next examined human brain-derived parkin by LC-MS/MS. Here, we mapped cysteines in both reduced and oxidized states for the 52.5 kDa monomer immunopurified from control cortices. The latter included irreversible oxidation events, such as to sulfonic acid at C253 (**Fig. 2.2g-i**) and C95 (**Fig. 2.2j,k**), as well as reversible ones, such as at C253 (**Extended Data Table 2.1**).





Extended Data Figure 2.4: Oxidation of parkin's thiols promotes insolubility.

(a-d) Detection of r-parkin in soluble (supernatant) and insoluble phases (pellet; recovered by 10% SDS-containing buffer) following exposure to increasing concentrations of (a) aminochrome (0-200 μM), (b) CCCP (0-200 μM), (c) rotenone (0-2mM) and (d) DTT (0-1M) as described in Fig. 2.2a.

(e-f) Circular dichroism spectra of H₂O₂-exposed WT r-parkin secondary structure at (e) T=0 (soluble fraction) and at (f) T=5 days of incubation at 37° C for both soluble (black line) and aggregated (red line) states as described in Fig. 2.2c,d.

(g) Dynamic light scattering analysis showing progressive size changes, as measured in hydrodynamic diameters (nm), was monitored during 0, 1, 3 and 5 hrs at room temperature. The structural state for wild-type, r-parkin under non-reducing, native conditions showed increased aggregate formation over time, which was partially reversed by treatment with DTT as indicated.

(h) Summary of LC-MS/MS based analysis of cysteine oxidation state of untreated and H₂O₂-treated r-parkin as indicated using IAA-DTT-NEM fingerprinting to identify reduced cysteines (IAA) or reversibly-oxidized residues (NEM). For comparison, see Extended Data Table 2.1 for modified cysteines identified in parkin purified from human brain cortex. A complete list of modified cysteines under each condition tested is found in Supplemental Information Table 2.2.

	TS-extractable (IP, n=4) Mean (range)	SDS-extractable (IP, n=8) Mean (range)
% Parkin protein coverage	46.8 (35-72)	19.4 (2-58)
# Peptides identified	15.0 (10-23)	6.4 (1-17)
# Cysteines identified	17.5 (13-26)	6.1 (1-16)
Reduced residues (IAA-Cys)	17.5 (13-26)	6.1 (1-16)
IAA/Total cysteines	17.5/35	6.1/35
%	50	17.5
IAA/Total identified cysteines	17.5/28	6.1/28
%	62.5	21.8
IAA/Total cysteines per run	17.5/28	6.1/16
%	62.5	38.1
Oxidized residues	Among PTMs preselected for screening	Among PTMs preselected for screening
Cys --> Dha	Cys 95, 196, 238, 241, 253	Cys 196
NEM-cysteine	Not detected	Cys 253
Sulfinic acid - Cys dioxidation	Not detected	Not detected
Sulfonic acid - Cys trioxidation	Cys 95, 253	Not detected
Glutathionylation	Not detected	Not detected
S-nitrosylation	Not detected	Not detected
Dopamine quinone adduct formation	Not detected	Not detected
Methionine oxidation	Met 80, 192, 327, 458	Met 80, 192, 327, 458

Extended Data Table 2.1: Summary of LC-MS/MS results with a focus on parkin modifications in human brain.

Results for twelve IP runs (TS extracts; n=4; SDS extracts, n=8) of human cortices to identify reduced (IAA-tagged) and reversibly-oxidized (NEM-tagged) thiol residues. Parkin immunoprecipitation (IP) from human brains was performed using magnetic beads conjugated to the Prk8 antibody or a parkin monoclonal antibody (Clone B). Tissue extraction and IP were performed in the presence of IAA. Higher parkin coverage was consistently obtained from the TS-extracted fractions, along with a higher number of IAA-cysteines/total cysteines identified per run, when compared to the SDS-soluble fractions. In addition to IAA and NEM, we pre-selected to identify other non-reversible post-translation modifications for screening as indicated. In human cortices, we detected cysteines irreversibly oxidized to sulfonic acid (trioxidation), e.g., at C95 and C253 and NEM-labelled, reversibly oxidized cysteine, C253. Among the pre-selected PTMs we also detected cysteine to dehydroalanine (Cys → Dha) shift and methionine oxidation.

Parkin reduces H₂O₂ to water

We next asked whether parkin's own oxidation had reducing effects (**Fig. 2.3**; **Extended Data Fig. 2.5a-f**). Using r-parkin *in vitro*, we discovered that it directly lowered H₂O₂ levels in a time- and concentration-dependent manner (**Fig. 2.3a**; **Extended Data Fig. 2.5c**). This reaction (*e.g.*, $2\text{R-SH} + \text{H}_2\text{O}_2 \rightarrow \text{R-S-S-R} + 2\text{H}_2\text{O}$) was dependent on thiol integrity, because pre-treatment with IAA abolished it (**Fig. 2.3b**). Parkin's reducing capacity was confirmed in parallel studies employing a different H₂O₂ assay³⁴⁹.

We also examined other proteins, *e.g.*, r-DJ-1, a C-terminal RING2 peptide (r-parkin_{321C}), and two ARPD-linked mutants, p.G328E and p.C431F (**Fig. 2.3c-d**, **Extended Data Fig. 2.5d-f**). There, r-DJ-1 and r-parkin_{321C} showed negligible H₂O₂-reduction, and the two point mutants conferred less activity than wild-type r-parkin (**Fig. 2.3c**; **Extended Data Fig. 2.5d,e**). These reducing effects correlated with r-parkin's own oxidation, as revealed by SDS-PAGE after the reaction (**Fig. 2.3d**). We speculated that this anti-oxidant activity was dependent on the reactive thiol content, which we tested using the Ellman's reagent. There, wild-type r-parkin, r-parkin_{321C} and r-DJ-1 showed the predicted number of reactive thiols, whereas the point mutants had fewer accessible thiols (**Fig. 2.3e**). The correlation between thiol equivalencies and H₂O₂-reduction was linear (**Fig. 2.3f**).

H₂O₂ is elevated in parkin-deficient brain

To explore whether parkin oxidation conferred ROS reduction in human brain, we quantified H₂O₂ levels in cortices of ARPD patients *vs.* age-, *post mortem* interval-, ethnicity- and brain region-matched control specimens^{193,252}. There, we found elevated

H₂O₂ concentrations in parkin-deficient brain (P<0.05; **Fig. 2.3g**). Specimens of non-*PRKN*-linked cases with parkinsonism showed H₂O₂ levels comparable to age-matched controls (**Fig. 2.11**). We concluded that parkin actively contributes to ROS reduction in human brain. In related mouse experiments, we determined that parkin regulates H₂O₂ concentrations in the cytosol³⁴⁹.

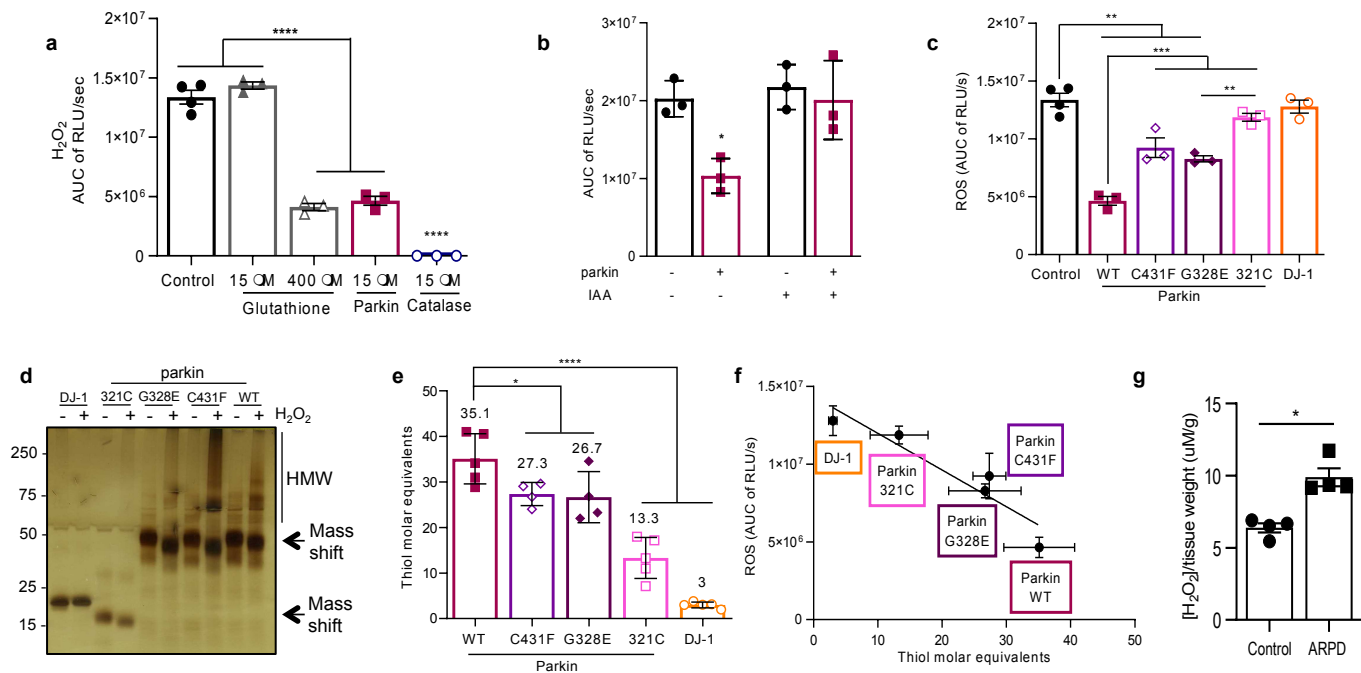


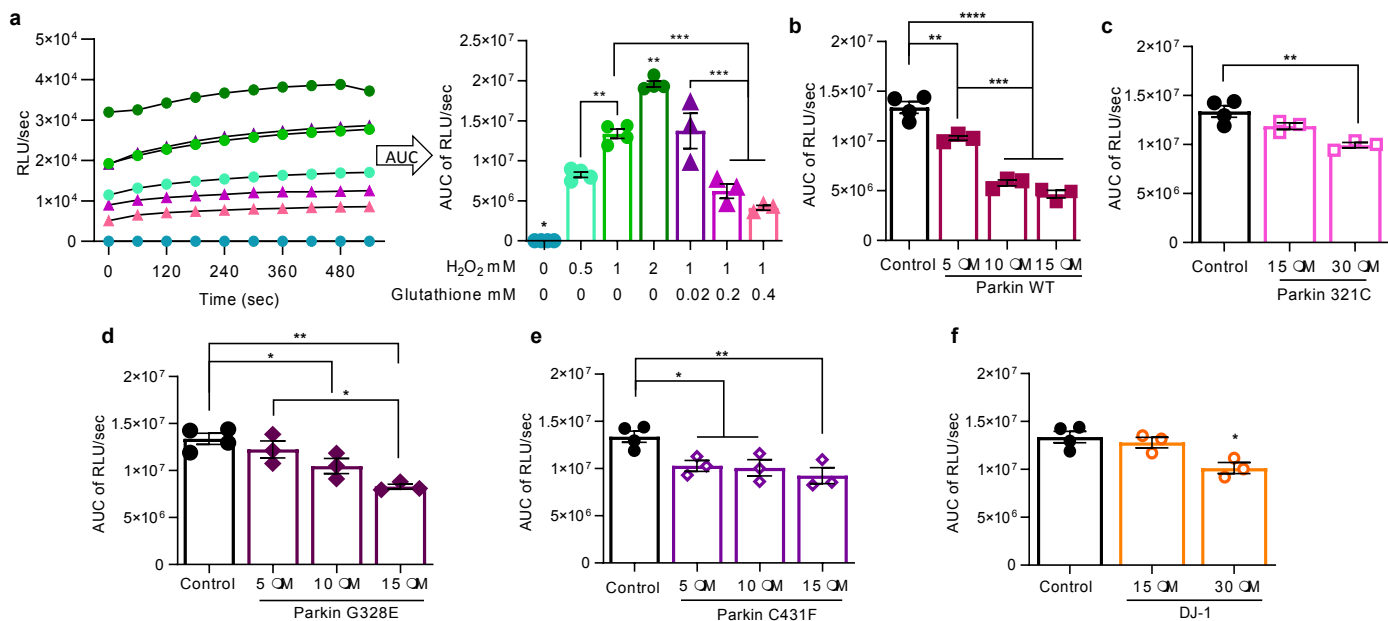
Figure 2.3: Parkin directly reduces H_2O_2 to water.

(a-d) Area under the curve (AUC) plots for results from *in vitro* colorimetric H_2O_2 assays, where AUC integrates total H_2O_2 present over the time course of the assay (see also **Extended Data Fig. 2.5a**). (a) Both full-length r-parkin and glutathione significantly reduced H_2O_2 ; (b) This was blocked when r-parkin was pre-incubated with IAA. (c) Comparison of WT r-parkin with DJ-1, two r-parkin point mutants, and r-parkin₃₂₁₋₄₆₅ (321C). Results represent $n=3 \pm \text{SD}$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ using one-way ANOVA with Tukey's post hoc test. (d) Visualization of proteins post H_2O_2 exposure by silver staining (non-reducing conditions).

(e) Quantification of reactive thiol content (in molar equivalents) of r-parkin (WT; point mutants; 321C) and full-length r-DJ-1 using the Ellman's reagent assay.

(f) Correlation curve between number of free thiols (e) and H_2O_2 reducing capacity (c) for indicated proteins.

(g) Quantification of H_2O_2 in human brain from *PRKN*-deficient ARPD cortices compared to age- and *post-mortem* interval-matched controls collected at the same institution. Results are represented as the mean concentration of H_2O_2 per g of tissue analyzed ($\mu\text{M/g}$; $n=4/\text{group}$) $\pm \text{SEM}$; * $p < 0.05$ determined using Student T-test.



Extended Data Figure 2.5: Parkin directly reduces H₂O₂ to water in a concentration-dependent manner.

(a) Kinetic readings from *in vitro* colorimetric H₂O₂ assays (left panel) comparing increasing concentrations of input H₂O₂ (green lines) and the effect of increasing concentrations of glutathione (purple, pink lines). Curves were converted to the area under the curve (AUC) where AUC integrates the total value of H₂O₂ signals generated over the 10 minutes time course of the assay (right panel).

(b-f) AUC graphs for results from *in vitro* H₂O₂ assays for various concentrations of recombinant proteins, as indicated. Statistical analysis was performed as in Fig. 3c.

Parkin prevents dopamine toxicity in part by lowering H₂O₂

To explore its selective neuroprotection, we revisited parkin's role in DA toxicity studies²⁶⁰. We first tested its effect on redox homeostasis using human DA-ergic M17 cell cultures. Here, wild-type parkin, but not ARPD mutants p.G328E and p.C341F, protected cells from rising DA concentrations (**Fig. 2.4a; Extended Data Fig. 2.6a**). Moreover, DA exposure resulted in a rise in endogenous H₂O₂ concentrations, which wild-type -but not mutant- parkin reversed (**Fig. 2.4b**). These results validated our *in vitro* and *in vivo* findings (Fig. 2.3). In stable M17 cells, protection from DA toxicity paralleled *PRKN* -cDNA expression levels (**Fig. 2.4c**); approximately 4 ng of parkin neutralized each mM of DA. Neuroprotection also correlated with parkin's own insolubility due to oxidation. These changes were not reducible by DTT (**Fig. 2.4d,e**), indicating irreversible thiol modifications.

Parkin binds dopamine radicals at cysteine 95

We asked whether parkin aided in the neutralization of DA radicals through direct conjugation at specific residues²⁶⁰. Full-length r-parkin, which carried DA and aminochrome adducts (as shown by nitro-blue-tetrazolium staining; **Fig. 2.4f**), was analyzed by LC-MS/MS. There, we identified several aminochrome, DA and indole-5,6-quinone adduct-carrying peptides specific to treated r-parkin (**Extended Data Fig. 2.6c,d**). Intriguingly, the most frequently modified residue was C95, a primate-specific cysteine of parkin (**Fig. 2.4g; Extended Data Fig. 2.6c-e**).

Parkin augments melanin formation

Given the observed interactions between r-parkin, DA radicals and aggregate formation, we examined whether melanin formation was altered. Polymerization of DA into melanin occurs in a spontaneous, oxidation-dependent manner via intermediates, such as indole-5,6-quinone, which is blocked by glutathione^{53,350}. We discovered that wild-type parkin and both point mutants tested augmented melanin formation *in vitro* in a time- and concentration-dependent manner (**Fig. 2.4h; Extended Data Fig. 2.6b**). We next sought to test parkin's involvement in melanin formation *in vivo*.

Parkin colocalizes with neuromelanin in adult *S. nigra* neurons

To visualize parkin's subcellular localization in adult brain, we developed several, monoclonal antibodies to human parkin suitable for microscopy; these were epitope-mapped and validated regarding their specificity (*e.g.*, **Extended Data Fig. 2.7**). When staining serial sections of control midbrains, three clones revealed intense, granular parkin staining in the cytoplasm of pigmented neurons from adults (ages, ≥ 55 years), albeit at varying intensities (clones-E, -D, -G; **Extended Data Fig. 2.8a**). We counted anti-parkin reactivity in >83% of anti-tyrosine hydroxylase-positive cells and -to a lesser extent- in non-pigmented neurons of normal midbrain and cortex (**Extended Data Fig. 2.8b,c**).

By immunohistochemistry, in *S. nigra* neurons anti-parkin signals strongly overlapped with neuromelanin pigment (**Extended Data Fig. 2.8d**). Sections from individuals with Parkinson's (not linked to *PRKN* mutations) showed similar anti-parkin reactivities in

surviving neurons and overlapping with extracellular pigment. We found much less anti-parkin reactivity in nigral neurons of younger subjects (ages, ≤ 33 years), which matched their paucity of neuromelanin content (**Extended Data Fig. 2.8d,e**).

Parkin localizes to LAMP-3⁺-lysosomes within nigral neurons

In screening organelle markers, we detected highly similar staining between antibodies to parkin and lysosome-associated protein-3 (LAMP-3/CD63; **Extended Data Fig. 8f**). Intriguingly, variants at the *LAMP-3* locus have recently been linked to PD risk³⁵¹. Under these conditions, antibodies to LC3B (but not to mitochondrial antigens MnSOD and VDAC) generated some signal overlap with anti-parkin (not shown).

Because neuromelanin granules are thought to arise within lysosomes^{Zucca, 2018}, we also carried out immunofluorescence microscopy. There, antibodies to parkin and LAMP-3 strongly co-labelled with pigmented granules (**Fig. 2.4i**). Of note, LAMP homologues have recently been shown to promote long term storage of melanin in human keratinocytes³⁵².

Using confocal microscopy, we demonstrated that in adult midbrain anti-parkin signals and neuromelanin granules were frequently surrounded by circular anti-LAMP-3 reactivity (**Fig. 2.4j**). A z-stack video for the parkin-LAMP-3 co-labelling studies is appended (Supplemental Information). We concluded that in normal human midbrain parkin is associated with LAMP-3-mediated sequestration of neuromelanin.

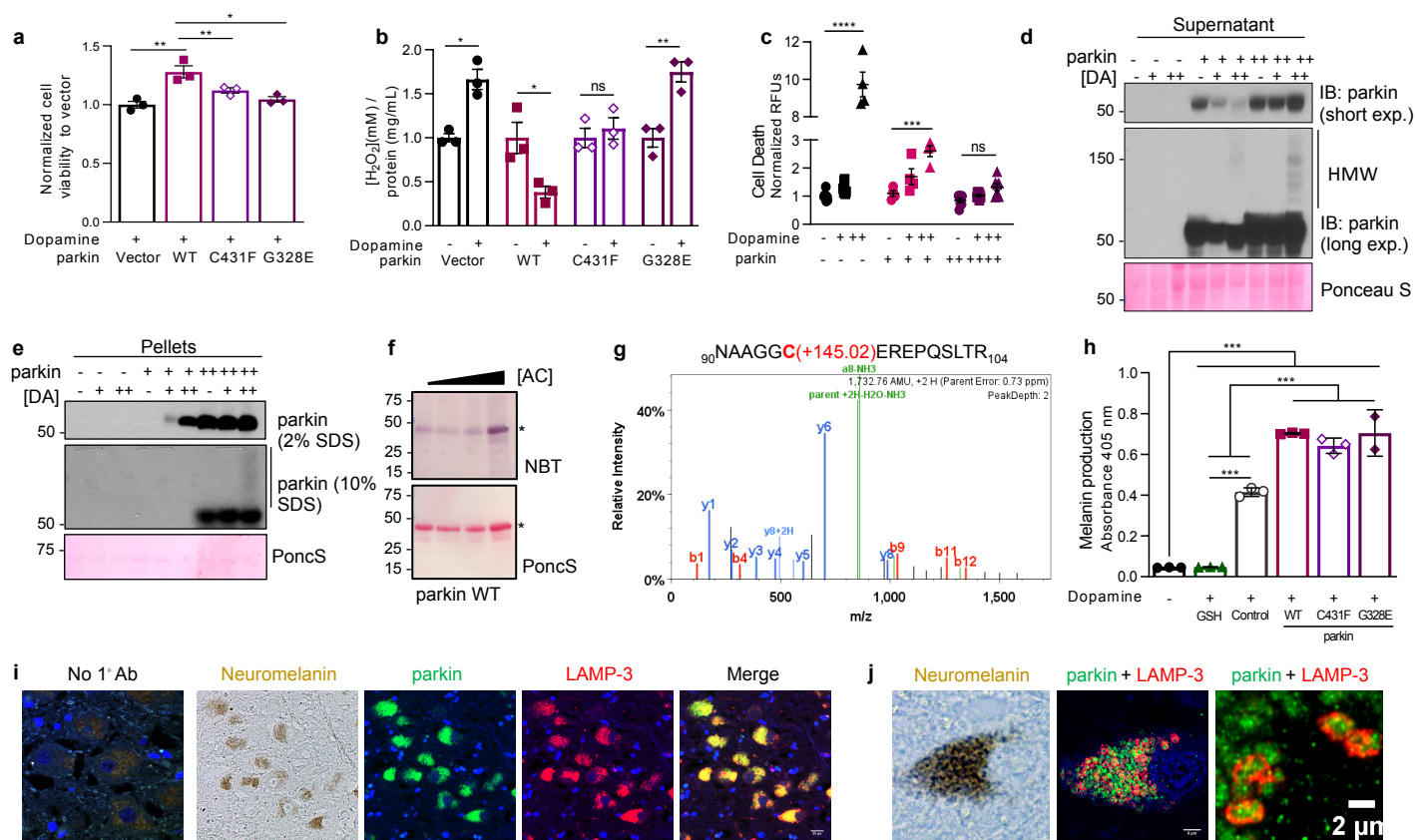


Figure 2.4: Human parkin conjugates dopamine radicals and promotes melanin formation.

(a-b) Cell viability assay **(a)** and **(b)** H_2O_2 quantification for dopamine-treated M17 cells expressing WT or two ARPD-linked parkin mutants. Cells were exposed to 200 μ M dopamine or vehicle for 20h as indicated. Data points represent the mean of duplicates $\pm$ SEM. * p <0.05 by 1-way ANOVA; (n=3 experiments).

(c) Cell viability assay of dopamine-treated human M17 neuroblastoma cells that stably express vector-control plasmid (parkin -) or human *PRKN* cDNA at mid- (+) or high (++) levels. Cells were exposed to 20 μ M (+) and 200 μ M (++) dopamine for 20h. Representative data are shown for the mean of duplicates $\pm$ SEM from n=4-8 independent experiments; * p <0.05 by 1-way ANOVA.

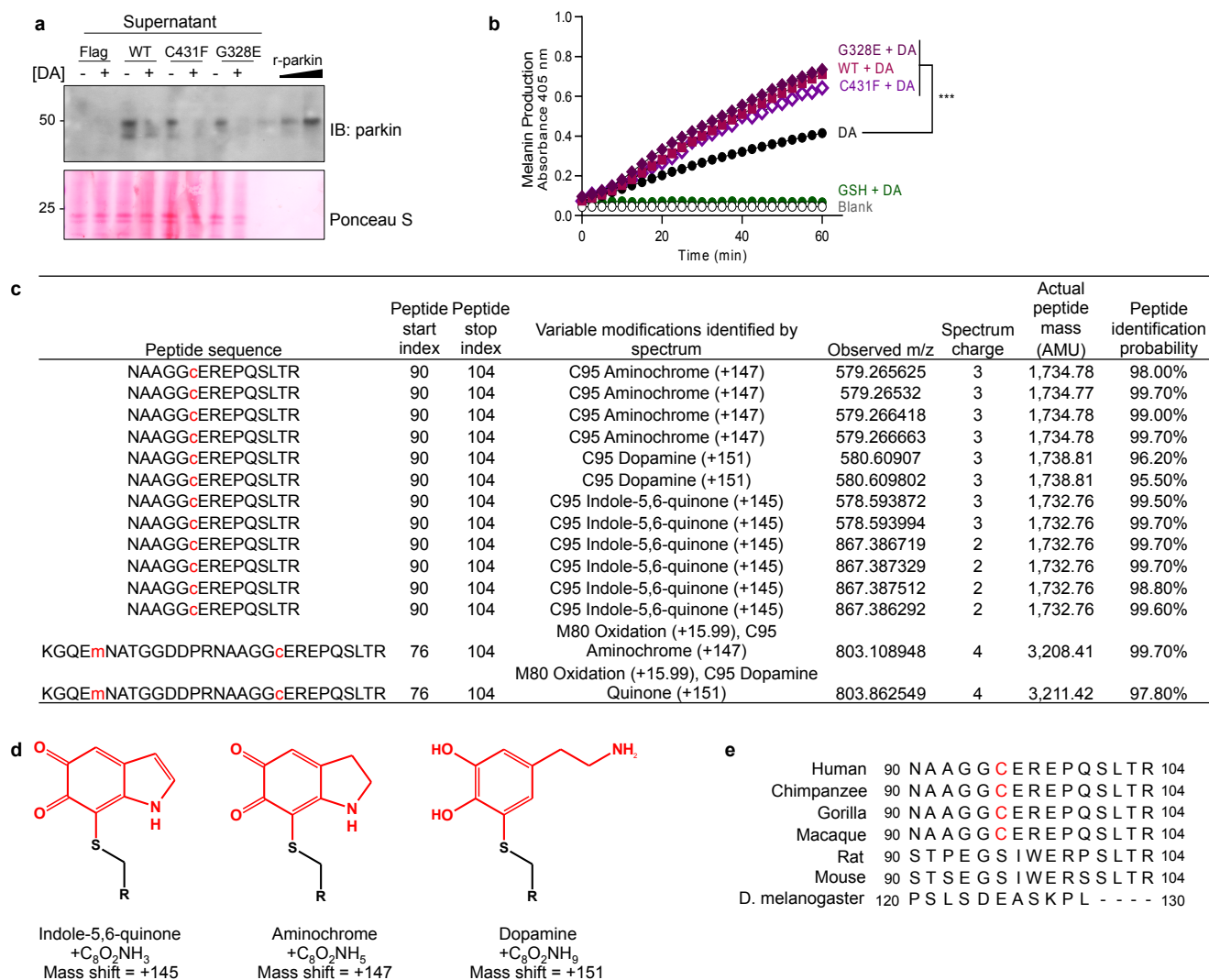
(d-e) Western blots of parkin in soluble (supernatant, **d**) and insoluble (serial pellets, **e**) fractions of lysates described in **(c)**. SDS/PAGE was run under reducing conditions.

(f) Nitro blue tetrazolium (NBT) and Ponceau S staining of WT human r-parkin that had been incubated with increasing concentrations of aminochrome *in vitro*; gel was run under reducing conditions. *Denotes parkin monomers.

(g) LC-MS/MS-generated spectra of trypsin-digested human r-parkin peptide aa90-104 carrying an indole-5,6-quinone adduct (+145 mass gain) at C95 (Mascot ion score 31.1). See also **Extended Data Fig. 2.6c,d**.

(h) AUC of kinetic result of melanin formation in the presence or absence (blank) of WT r-parkin, two point mutants and glutathione (GSH) using a colorimetric assay. Data represent the mean of triplicates $\pm$ SEM. *** p <0.05 by 1-way ANOVA. See also **Extended Data Fig. 2.6b**.

(i-j) Microscopy signals generated by double labelling of human *S. nigra* dopamine neurons, using anti-parkin (clone-E; green colour) and anti-LAMP-3/CD63 (red colour) antibodies; (blue colour, Hoechst stain). Light microscopy images in the same field (brown neuromelanin pigment is visible; second panel) and no primary Ab control run in parallel to experiment (left panel) are shown. Higher magnification of individual dopaminergic neuron is shown in **(j)**.



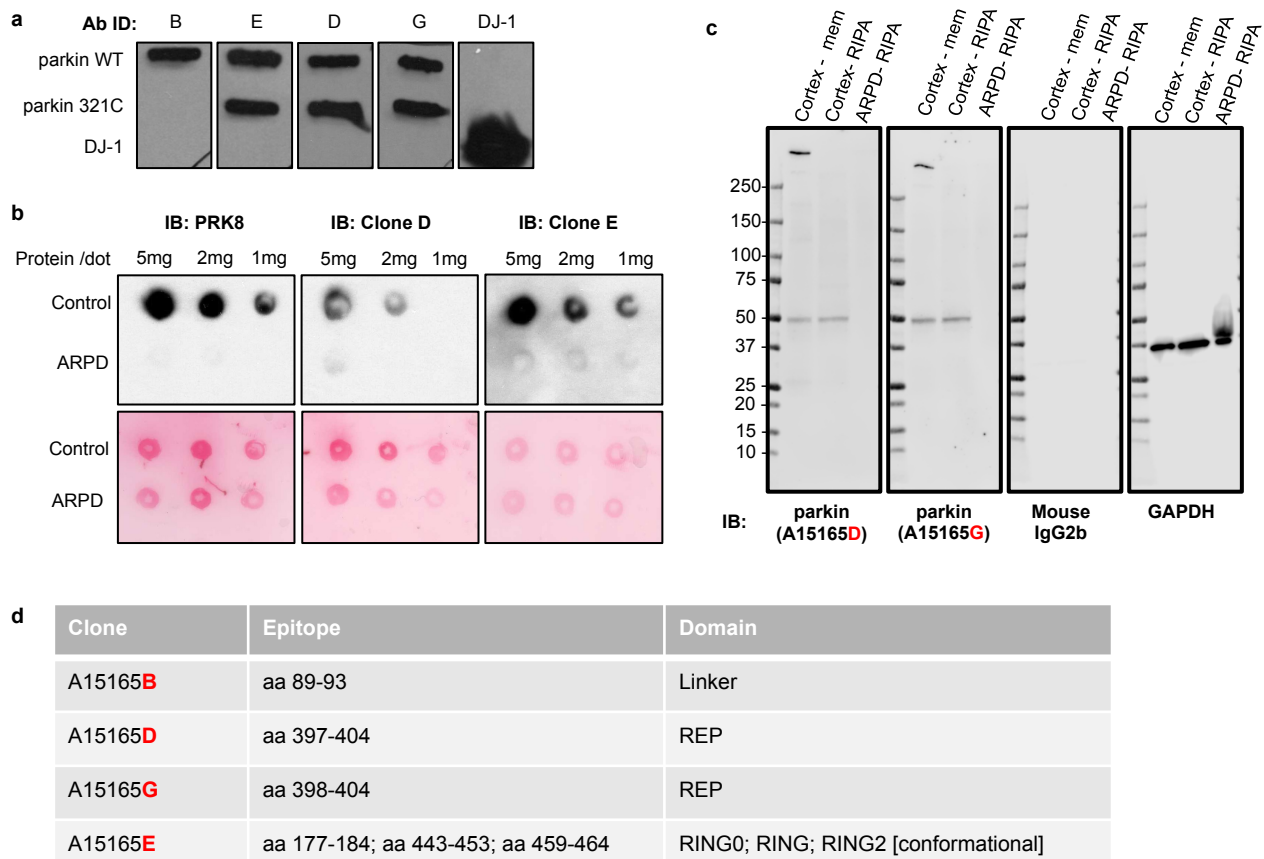
Extended Data Figure 2.6: Human parkin conjugates dopamine metabolites at C95.

(a) Western blot analysis of ectopic, WT and mutant parkin expressed in dopamine-treated M17 cells, as described in Fig. 2.4a,b.

(b) *In vitro* melanin formation, as monitored by a colorimetric assay (absorbance rate, 405nm) demonstrating increased polymerization of dopamine (DA) over a 60 min period in the presence or absence (blank) of wild-type (WT) r-parkin and two point mutants (G328E; C431F). Melanin synthesis from dopamine is blocked by glutathione (GSH). Data represent the mean of triplicates $\pm$ SEM. *** $p < 0.05$ by 1-way ANOVA.

(c) Table summarizing LC-MS/MS-based detection of adducts of dopamine-metabolites conjugated to C95 of r-parkin following exposure to aminochrome. Chemical structures for identified cysteine-conjugated adducts are shown in (d).

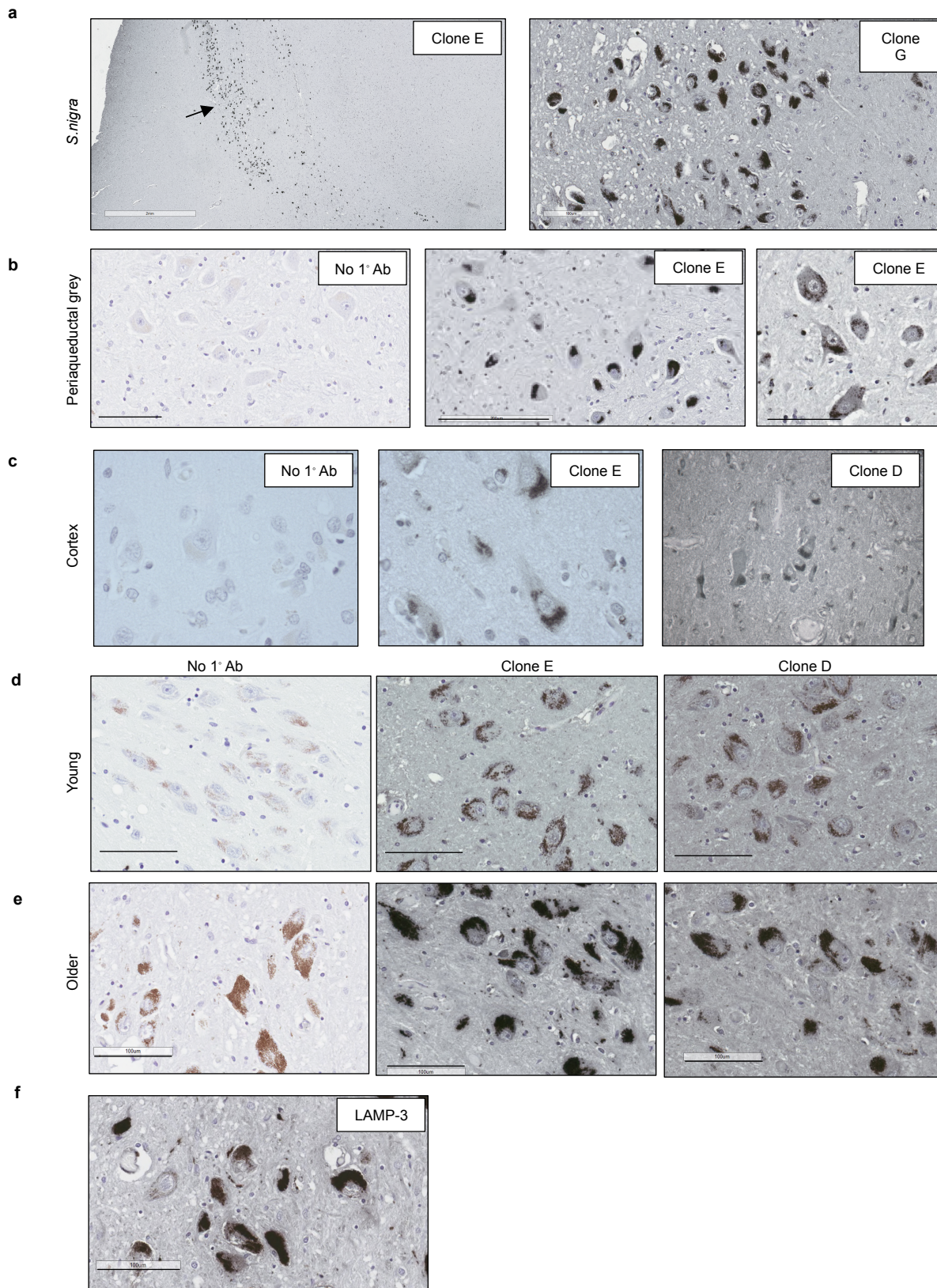
(e) Species sequence alignment for parkin peptide aa90-104, C95 is highlighted in red.



Extended Data Figure 2.7: Characterization of four, new monoclonal antibodies to human parkin.

(a-c) Characterization of four murine, monoclonal antibodies (of IgG₂ isotype; clone-B, -E, -D, and -G) in three different assays: (a) non-denaturing slot blots (100ng/slot) against wildtype full-length (WT) or the C-terminal peptide (aa321-465; 321C) r-parkin, and human r-DJ-1; (b) non-denaturing dot blots against human brain lysates (SDS fractions from control and *PRKN*-linked ARPD cases); and (c) by denaturing SDS/PAGE (reducing conditions) and Western blotting of extracts from cortical specimens of a control brain and a parkin-deficient ARPD case. Screening by these three methods as well as by cell-based microscopy (using indirect immunofluorescence, not shown) revealed specific staining for four anti-parkin clones (-B, -E, -D and -G), which was conformation-dependent for clone-E.

(d) List of epitopes within the sequence of human parkin, as recognized by clones -B, -E, -D, and -G and identified by screening with overlapping 7-12 amino acid-long peptides covering full-length, human parkin. Note, the clone E epitope is conformational, comprised of the three regions as indicated.



Extended Data Figure 2.8: Parkin is detectable in human brain sections by routine microscopy.

Immunohistochemical detection of parkin in ageing human brain including **(a)** dopaminergic neurons of the *S. nigra*, **(b)** periaqueductal grey neurons in the posterior midbrain and **(c)** cortex, as well as in the *S. nigra* from adult control subjects aged **(d)** 24 and **(e)** 66 years. For all panels, parkin reactivity was detected using the indicated clones and metal-enhanced DAB (black colour). No primary antibody (Ab) controls are shown. Scale bars represent 100 μ m, or as indicated. **(f)** Immunohistochemical detection of LAMP-3 protein in dopaminergic neurons of the *S. nigra* from an adult control brain.

Discussion

We identified a heretofore unrecognized role for parkin in redox homeostasis. To our knowledge, only one other protein has a similar duality in functions (*i.e.*, ubiquitylation and redox biology), namely the sensitive-to-apoptosis gene product, SAG (aka RBX2/ROC2/RNF7)³³⁵. Our work establishes that parkin's progressive insolubility in human midbrain, first described in the cortex by Pawlyk et al.³³², is due to oxidative modifications during ageing (**Fig. 2.1, 2.2d-k; Extended Data Table 2.1**). Unlike in human brain, in skeletal muscle ~50% of detectable parkin remains soluble. In all other aged mammals we examined, brain parkin remains soluble to an even greater degree (**Fig. 2.1i; Extended Data Fig. 2.1.e,f**). Theoretically, other modifications (*e.g.*, phosphorylation²⁷¹) could also alter parkin's solubility.

Heretofore, parkin oxidation has been widely interpreted as a 'loss-of-function' *in vivo*. In contrast, we posit that its oxidative modifications reflect parkin's physiological role in maintaining redox balance. However, ubiquitylation and redox stabilization may not be mutually exclusive. Low concentrations of pro-oxidants can activate parkin's E3 activity *in vitro*²⁵⁸, which we confirmed.

The cellular thiol network is critical in neural health³⁴⁰. As summarized in **Extended Data Fig. 2.9**, through its oxidation we postulate that parkin neutralizes ROS (*e.g.*, H₂O₂; **Fig. 2.3**), reduces RNS, conjugates RES (*e.g.*, quinones), promotes melanin formation, and sequesters radicals within aggregates (**Fig. 2.4**). This multimodal redox function is supported three-fold. One, in mice by evidence of oxidative stress in parkin's

absence^{94,95}. Two, in humans by related findings in ARPD brain (**Fig. 2.3**) and of lesser neuromelanin content in surviving DA neurons^{207,208,210,211,353,354}. We noted with interest that primate-specific residue C95 was most consistently found to conjugate DA radicals (**Fig. 2.4g; Extended Data Fig. 2.6c,e**). And three, through parallel work, we discovered a dynamic interplay between *PRKN* expression and glutathione *in vivo*³⁴⁹.

We found that single point mutants have measurable redox effects depending on the paradigm tested (**Figs. 2.3, 2.4**). Complementary studies will inform clinical genotype-to-phenotype studies in humans^{201,202}. Several reports suggested that point mutation carriers may have a later age-of-onset of ARPD than those whose *PRKN* alleles encode no RING domain^{201,202}. The translocation of parkin to mitochondria and lysosomes could be facilitated by oxidation-induced multimer formation (**Fig. 2.2g,h**). The concept of ‘RING scaffolding’ has previously been described for other ubiquitin ligases³⁵⁵. Because neuronal health is co-regulated by thiol chemistry³⁵⁶, we posit that parkin confers its ‘inherently present, pro-mitochondrial benefit’²⁸⁹ through redox homeostasis.

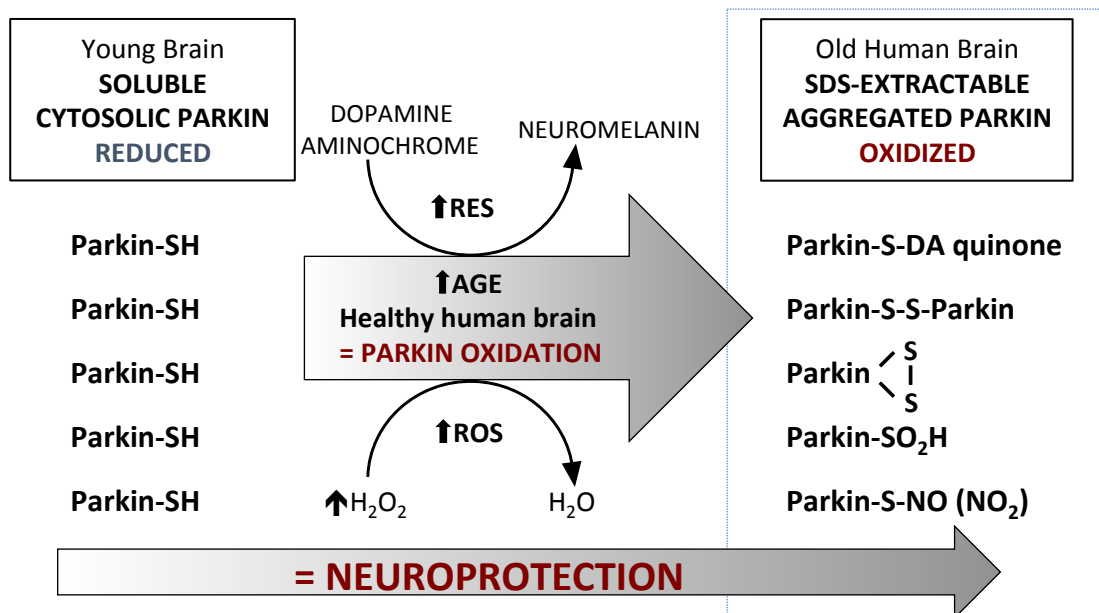
Our study raises new questions. Beyond its co-localization with neuromelanin in LAMP-3⁺-positive lysosomes (**Fig. 2.4i,j**), our results have not yet revealed the fate of oxidized parkin. Human LAMP proteins store melanin in keratinocytes protecting them from UV light³⁵². Parkin oxidized by ROS and RES together with LAMP-3 could play a similar role in the storage of neuromelanin. Other radicals could be similarly neutralized by parkin in other regions of adult human brain. Of note, the three clones that detect parkin by microscopy seem to preferentially bind to aggregated forms *in situ*; one has cysteines

within its epitopes (clone-E; **Extended Data Fig. 2.7**). Little anti-parkin signal was detected in midbrain sections from young adults (**Extended Data Fig. 2.8d**), whereas the holoprotein was readily detected by immunoblotting (*e.g.*, **Fig. 2.1a**).

Several limitations were identified. One, although we have detected the *PRKN* gene to be continuously transcribed in nigral neurons (**Fig. 2.1e**), the rate of mRNA translation under redox stress remains to be studied. Future studies will employ sensitive ELISAs to quantify parkin in tissue lysates. Two, we have only begun to map parkin's oxidative modifications in normal human brain (**Extended Data Table 2.1**). As highlighted in **Extended Data Fig. 3**, parkin likely carries a heterogeneous set of cysteine modifications in human brain, making qualitative mapping by LC-MS/MS difficult. Moreover, ROS and RNS modifications can be very transient and thus difficult to capture directly^{256,258,357}. Three, we have not yet completed a quantitative assessment of oxidative modifications in young vs. old brains. Future MS studies require further optimization and larger amounts of tissues including from healthy and diseased midbrains. In addition, access to organoids³⁵⁸, specifically those generated from subjects with *PRKN* mutations, will help clarify which parkin domains participate in neuromelanin formation.

The strength of this study is that it focused on parkin metabolism in human brain. *PRKN*-linked ARPD is a human condition, and we are guided by insights from human studies. We have shown that parkin fulfils three criteria of a typical redox molecule: the sensing of stressors; structural responses to them; and the participation in corrective measures. If confirmed by future work, this new concept for parkin's neuroprotective function in human brain opens the door to explore its possible role in non-*PRKN*-linked PD and

parkinsonism associated with advanced age. It also allows the reprioritization of therapeutic avenues in patients afflicted by ARPD, such as through gene therapy⁸ and polyvalent, anti-oxidant therapy³⁵⁹.



Extended Data Figure 2.9: Proposed model for parkin's redox functions in human midbrain.

In human brain, parkin thiol (-SH) oxidation neutralizes cellular reactive oxygen species (ROS; H₂O₂) and potentially toxic dopamine (DA) radicals (DA quinones; RES) during normal ageing. The oxidation of wild-type parkin promotes higher molecular weight species formation, insolubility and aggregation. Both reversible and irreversible oxidation events occur, which promote its transition into an insoluble, aggregated state by the beginning of the 5th decade. The oxidation and aggregation lead to parkin's accumulation within LAMP-3-positive lysosomes within DA neurons of the *S. nigra*. The multimodal oxidation of parkin confers neuroprotection. In *PRKN*-linked ARPD, the absence of parkin's redox effects leads to a rise in ROS, reduced sequestration of DA radicals and less neuromelanin formation.

Materials And Methods

Tissue collection

All tissue was collected in accordance with Institutional Review Board-approved guidelines. Fresh frozen samples of cortical human brain from subjects under 50 years of age were acquired through the University of Alabama and the Autism Tissue Program. *Post mortem*, frozen brain samples from frontal cortices were also obtained from the NICHD Brain and Tissue Bank at the University of Maryland. Brain tissues, including midbrain specimens, with short PMI were also obtained from patients diagnosed with clinical and neuropathological MS according to the revised 2010 McDonald's criteria (n=4)³⁶⁰. Tissue samples were collected from MS patients with full ethical approval (BH07.001) and informed consent as approved by the CRCHUM research ethics committee. Autopsy samples were preserved and lesions classified using Luxol Fast Blue / Haematoxylin & Eosin staining and Oil Red O staining as previously published^{361,362}. No inflamed tissue areas were used in this current study. Additional, fresh-frozen and paraffin-embedded human samples were obtained from the Neuropathology Service at Brigham and Women's Hospital and from archived autopsy specimens in the Department of Pathology and Laboratory Medicine of The Ottawa Hospital. Human spinal cord and muscle tissues were collected at The Ottawa Hospital with approval from the Ottawa Health Science Network Research Ethics Board.

Wild-type C57Bl/6J mice were used for analysis of the effects of *post mortem* interval on murine parkin in the brain. Mice from 4 to 8 months old were perfused with PBS and their brains were collected for PMI experiments.

Sequential extraction of parkin from tissue

Roughly 1 cm³ samples of human brain frontal cortex and midbrain (age range 5-85 years of age) were weighed and placed in 3X volume/weight of Tris Salt buffer (TSS) (5mM Tris, 140 mM NaCl pH 7.5) containing complete EDTA-free protease inhibitor cocktail, and 10 mM iodoacetamide (IAA). The samples were homogenized on ice in a Dounce glass homogenizer by 50 passes, transferred to ultracentrifuge tubes and spun at 55,000 rpm and 4°C for 30 mins. The TS supernatant was transferred to a fresh tube and the pellet was extracted further with addition of 3x volume/weight of Triton X-100 buffer (TX, TS + 2 % Triton X-100). The samples were mixed by vortex, incubated on ice for 10 min and centrifuged again using the same prior setting. The TX supernatant was transferred to a fresh tube and the pellet was extracted further with addition of 3x volume/weight of SDS buffer (SDS, TS + 2 % SDS). The samples were mixed by vortex, incubated at room temperature for 10 min and centrifuged again at 55,000 rpm and 12°C for 30 mins. The SDS supernatant was transferred to a fresh tube and the pellet was either stored at -80°C or extracted further with addition of 3X volume/weight of 6X non-reducing Laemmli buffer (LB, 30 % SDS, 60 % glycerol, 0.3 % bromophenol blue, 0.375 M Tris. pH 6.8, 100mM DTT), mixed by vortex and incubated at room temperature for 10 min. Samples were centrifuged again at 55,000 rpm and 12°C for 30 mins and the LB supernatant was transferred to a fresh tube. Extracted proteins from TS, TXS and SDS buffers including pellet (20-30 µg) and 10-20 µL of LB extracts were run on SDS-PAGE using reducing (100 mM dithiothreitol, DTT) and/or non-reducing (0 mM DTT) loading buffer. Following transfer to membranes, Ponceaus S staining was used to confirm loading, and samples were blotted for parkin (Biolegend 808503, 1: 5,000), DJ-1 (ab18257, 1: 2,000), α -synuclein (syn1 1:1,000 or MJFR1 1:2000), LC3B (3868 1:2000),

VDAC (MSA03 1:5000), MnSOD and GLO1 (1:1000), calnexin (MAB3126), cathepsin D (sc-6486), GRP75 (sc-1058) . ImageJ software (1.52 k, National Institutes of Health, USA) was used for signal quantification purposes.

mRNA Analysis

PRKN mRNA isolated from individual *S. nigra* DA neurons (SNDA), cortical pyramidal neurons (PY) and non-neuronal, blood mononuclear cells (NN) were processed, as described³⁶³ and as annotated in the Human BRAINcode database (www.humanbraincode.org).

ROS (H₂O₂) measurements

Amplex[®] red hydrogen peroxide/peroxidase assay kit (Invitrogen A22188) was used to monitor endogenous levels of H₂O₂. Pre-weighed cortex pieces from human brains were homogenized on ice in the 1x reaction buffer provided, using a Dounce homogenizer (3 times volume to weight ratio). Homogenates were diluted in the same 1x reaction buffer (10x and 5x). A serial dilution of the H₂O₂ standard provided was prepared (20, 10, 2 and 0 μM). 50 μL of standards and samples were plated in a 96 well black plate with clear flat bottom. The reaction was started by the addition of 50μL working solution which consist of 1x reaction buffer, Amplex[®] red and horseradish peroxidase. The plate was incubated at room temperature for 30 minutes protected from light. A microplate reader was used to measure either fluorescence with excitation at 560 nm and emission at 590 nm, or absorbance at 560 nm. The obtained H₂O₂ levels (μM) were normalized to the tissue weight (g).

Recombinant protein expression in pET-SUMO vector

Wild-type and truncated (residues 321-465) human parkin were expressed as 6His-Smt3 fusion proteins in *Escherichia coli* BL21 (DE3) Codon-Plus RIL competent cells (C2527, New England Biolabs) as previously described^{234,364,365}. DJ-1 and SNCA coding sequences were cloned from a pcDNA3.1 vector into the pET-SUMO vector using PCR and restriction enzymes. ARPD-associated parkin mutants in the pET-SUMO vector were generated using site-directed mutagenesis. All proteins were overexpressed in *Escherichia coli* BL21 Codon-Plus competent cells (C2527, New England Biolabs) and grown at 37 °C in 2 % Luria Broth containing 30 mg/L kanamycin until OD600 reached 0.6, at which point the temperature was reduced to 16°C. All Parkin-expressing cultures were also supplemented with 0.5 mM ZnCl₂. Once OD600 reached 0.8, protein expression was induced with isopropyl β-D-1-thiogalactopyranoside, except ulp1 protease, which was induced once OD600 had reached 1.2. The concentration of IPTG used for each construct is as follows: 25 μM for wild-type and point mutants of parkin, and 0.75 mM for truncated parkin, DJ-1, α-synuclein, SAG, and ulp1 protease. Cultures were left to express protein for 16-20 h. Cells were then harvested, centrifuged, lysed and collected on Ni-NTA agarose beads in elution columns.

Protein redox chemistry and oxidative toxin conjugation

The recombinant protein samples were first prepared by removing excess TCEP, present in the elution buffer using repeat centrifugations (8 times 4000 x g at 4°C for 10 min) in Amicon Ultra 10kDa MWCO filters. The protein concentrations were measured and

adjusted to 20 μ M. Stock solutions of rotenone (1 mM), carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 50 mM), and hydrogen peroxide (H_2O_2 , 9.8 mM) were prepared. Aminochrome was freshly synthesized from Dopamine (see below). An aliquot of 10 μ L of each protein sample (at 20 μ M) was reacted with oxidants at the following concentrations: 0, 10, 20, 200, 2000 μ M rotenone; 0, 10, 20, 200 μ M CCCP; 0, 2, 20, 50, 200 aminochrome; 0, 20, 200, 500, 750, 1000, 2000 μ M H_2O_2 or 0, 10, 50, 100, 200, 500, 1,000 mM DTT. The samples were treated for 30 min at 37°C and centrifuged at 14,000 rpm for 15 min. The supernatant was transferred to a fresh tube and the remaining pellet was extracted with 10 μ L of T200-TCEP containing either 10 % SDS or 100 mM DTT. The pellets were incubated again for 30 min at 37°C and centrifuged at 14,000 rpm for 15 min. Laemmli buffer (10 μ L, containing 100 mM mercaptoethanol) was added to both the pellet and supernatant fractions and samples were separated on two SDS-PAGE. One gel was used for in-gel protein staining and the other was used for NBT staining. Specific bands of aminochrome treated wild-type full length r-parkin were excised from silver-stained gels and analyzed by LC-MS/MS as described below.

Aminochrome synthesis

A solution of 0.1 M sodium phosphate buffer pH 6.0 was prepared from a mixture of 12 mL of 1M NaH_2PO_4 and 88.0 mL of 1M Na_2HPO_4 . The reaction buffer (0.067 M sodium phosphate, pH 6.0) was prepared by adding 33 mL of 0.1 M sodium phosphate buffer to 17 mL water. A solution of 10mM dopamine in reaction buffer was prepared by adding 19 mg of dopamine hydrochloride to 1 mL of reaction buffer. Oxidation was activated by adding 5 μ L of tyrosinase (25,000 U/mL) and the mixture was incubated at room

temperature for 5 min. The tyrosinase was separated from the oxidized dopamine using a 50 kDa cut-off Amicon Ultra centrifugation filter by centrifuging at 14,000 rpm for 10 min. The absorbance of the filtrate was measured at a wavelength of 475 nm using Ultrospec 21000 pro spectrophotometer and the concentration of aminochrome was determined using the Beer-Lambert equation and extinction coefficient of $3058 \text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$.

Protein staining methods

All proteins were separated on pre-cast 4-12 % Bis-Tris SDS-PAGE gels (NPO321BOX, NPO322BOX, NPO336BOX) from Invitrogen using MES running buffer (50mM MES, 50mM Tris, 1mM EDTA and 0.1 % SDS, pH 7.3) and Laemmli loading buffer (10% SDS, 20% glycerol, 0.1% bromophenol blue, 0.125M Tris HCl, 200mM DTT or β -mercaptoethanol). Proteins were stained in gel using SilverQuest™ Silver Staining Kit (LC6070) from Invitrogen or Coomassie brilliant blue R-250 dye (20278) from ThermoFisher Scientific using the following protocol: The gel was transferred to a plastic container and rocked for 30 min in Fix Solution (10% acetic acid, 50% methanol), followed by staining for 2-24 h (0.25% Coomassie R250) until the gel turned a uniform blue. The stain was replaced with Destain Solution (7.5% acetic acid and 5% methanol) and the gel was rocked until crisp blue bands appeared. Following a wash with water the gel was stored in 7 % acetic acid. Proteins transferred to PVDF (1620177, Bio-Rad) membranes were stained with Ponceau S solution for 20 min, washed three times with water, imaged and then destained with 0.1M NaOH prior to Western blotting. Proteins conjugated to redox-cycling molecules were stained using the following NBT protocol:

Following Ponceau S staining and NaOH destaining, the membrane was washed twice with water, covered from light and incubated with rocking for 45 min in NBT solution [8-9 mg of NBT added to 14 mL of 2 M potassium glycinate buffer (75 g glycine in 0.4 L water and pH adjusted to 10.0 with KOH)]. The membrane was washed twice with destain solution (0.16M sodium borate) and incubated in fresh destain solution for 16-24 h. The membrane was washed once with water and imaged before drying.

Dynamic light scattering (DLS)

The protein buffer (50mM Tris, 200mM NaCl and 250 μ M TCEP, pH 7.5) was exchanged for a 20mM phosphate buffer with 10mM NaCl (pH 7.4). 20 μ M full-length wild-type r-parkin was centrifuged at 14,000 rpm for 60 min at 4 °C and light scattering intensity of the supernatant was collected 30 times at an angle of 90° using a 10 sec acquisition time. Measurements were taken at 37 °C using a Malvern Zetasizer Nano ZS instrument equipped with a thermostat cell. The correlation data was exported and analyzed using the nanoDTS software (Malvern Instruments). The samples were measured at 0-, 1-, 3- and 5 hours. Following 24 hr incubation, 2 mM DTT was added to the sample and the light scattering intensity of the supernatant was measured again.

Far UV circular dichroism spectroscopy

15 μ M of reduced and partially oxidized full-length wild-type r-parkin was measured at $t = 0$ and $t = 5$ days of incubation under native conditions in 20 mM phosphate, 10 mM NaCl buffer. The aggregates rich phase and the monomer rich phase in the samples were separated with ultracentrifugation (100,000 g for 2 hours). Far UV circular dichroism

(CD) spectra were recorded for the monomer and aggregated rich phase of protein samples using a JASCO J-720 spectrometer. The final spectrum was taken as a background-corrected average of 5 scans carried out under the following conditions: wavelength range 250–190 nm at 25 °C; bandwidth was 1 nm; acquisition time was 1 sec and intervals was 0.2 nm. Measurements were performed in a 0.01 cm cell. CD spectra were plotted in mean residue molar ellipticity units ($\text{deg cm}^2 \text{dmol}^{-1}$) calculated by the following equation: $[\Theta] = \Theta_{\text{obs}}/(10ncl)$, where $[\Theta]$ is the mean residue molar ellipticity as a function of wavelength, Θ_{obs} is the measured ellipticity as a function of wavelength (nm), n is the number of residues in the protein, c is the concentration of the protein (M), and l is the optical path length (cm). Secondary structure analysis of proteins using CD spectroscopic data was carried out using the BeStSel (Beta Structure Selection) software^{366,367}.

Chemiluminescence/ direct reactive oxygen species (ROS) assay

The assay was modified from Muller et al., 2013³⁶⁸ to measure the ROS-quenching ability of Parkin proteins, DJ-1, SNCA, BSA, GSH, and catalase. Protein concentrations were quantified using Bradford assay and adjusted to 5, 10, 15 and 30 μM in buffer not containing TCEP. BSA (10 and 20 μM), GSH (15, 20, 200, 400, 800 and 2000 μM), and catalase (0.015, 0.15, 0.25 and 15 μM) were prepared. Stock solutions of H_2O_2 for standard curve were prepared at 5, 10, 20, 40 and 50 mM in 0.1 M Tris HCl pH 8.0 using 30 % H_2O_2 . Stock solutions of 300 mM luminol and 40 mM 4-iodophenol were prepared in DMSO and protected from light. Signal reagent, containing 1.94 mM luminol and 0.026 mM 4-iodophenol, was prepared in 0.1 M Tris HCl pH 8.0 and protected from

light. A 0.4 % horseradish peroxidase solution was prepared using HRP-linked anti-rabbit secondary antibody diluted in Stabilizyme solution (SurModics SZ02). Each read was set up in triplicate on a white polystyrene 96-well plate (ThermoFisher 236105) and to each well was added 80 μ L Stabilizyme, 15 μ L of 0.4 % horseradish peroxidase (HRP) and 25 μ L of sample or controls. One of the injectors in a Synergy H1Multi-Mode Plate Reader (Bio Tek) was primed and set to inject 15 μ L of signal reagent and 15 μ L of each H_2O_2 stock solution was manually added to corresponding controls and samples just prior to reading. Final concentrations of reagents were 0.04 % HRP, 500, 1000, 2000, 4000 and 5000 μ M H_2O_2 , 194 μ M luminol, 2.6 μ M 4-iodophenol and 0.8, 1.7, 2.5 or 5 μ M of protein. The plate reader was set to measure luminescence every 1 min for a total of 10 min. The resulting kinetic data was converted to area under the curve (AUC) using Prism version 6. For samples pre-incubated with 20 mM iodoacetamide, a stock solution of 1 M iodoacetamide was prepared. To each well containing 25 μ L of sample, 0.52 μ L of 1 M iodoacetamide and 0.48 μ L of buffer not containing TCEP was added and the samples were incubated for 2 h at 37°C. Following incubation, the reagents for chemiluminescence were added as above except 79 μ L of Stabilizyme was used instead of 80 μ L and the samples were analyzed as above.

Thiol quantification of recombinant proteins

Recombinant protein samples were first prepared by exchanging the T200 protein buffer (50 mM Tris, 200 mM NaCl and 250 μ M TCEP, pH 7.5) for T200-TCEP using repeat centrifugations (8 times 4000 x g at 4°C for 10 min) in Amicon Ultra 10 kDa MWCO filters. The protein concentrations were measured and recorded. The glutathione stock

solution of 32,539 μM was prepared by dissolving 1 mg glutathione (GSH) in 1 mL of T200-TCEP and the standards 0, 50, 101, 203, 406, 813 and 1000 μM were prepared by serial dilution in T200-TCEP. The reaction buffer (0.1 M sodium phosphate, pH 8.0) was prepared by adding 93.2 mL 1M Na_2HPO_4 and 6.8 mL of NaH_2PO_4 in 1 L of water. Thiol detecting reagent (Ellman's reagent) was prepared by dissolving 2 mg of 5,5'-dithio-bis-[2-nitrobenzoic acid] (DNTB) in 1 mL of reaction buffer. The assay was performed in 96-well clear round bottom plates by adding 50 μL of thiol detecting reagent to 50 μL of sample or standard and incubating for 15 min at room temperature. The resulting 5-thio-2-nitrobenzoic-acid (TNB) produced was measured by absorbance at 412 nm using a Synergy H1Multi-Mode Plate Reader (Bio Tek). The amount of free thiols detected in each sample was calculated using the regression curve obtained from the glutathione standards and dividing by the concentration of the sample.

Cysteine labeling for mass spectrometry

The recombinant protein samples were first prepared by exchanging the T200 buffer for PBS. The protein concentrations were measured and adjusted to 10 μM using PBS. Stock solutions of 500 mM DTT, 100 mM iodoacetamide (IAA), 100 mM hydrogen peroxide and 250 mM ethylenediaminetetraacetic acid (EDTA) were prepared in PBS. A stock of 500 mM N-ethyl-maleimide (NEM) was prepared in ethanol immediately before use. For the first optimization and comparison of IAA and NEM labelling (*i.e.*, Table S2), r-parkin was treated with 2 mM DTT for 30 min at 37°C followed by incubation with 5 mM IAA or 85 mM NEM for 2 h at 37°C. The stepwise cysteine labeling procedure was as follows: A 10 μL aliquot of protein (at 10 μM) was reacted with hydrogen peroxide at

various concentrations, as indicated (Table 1) for 30 min (and up to 60 min) at 37°C as indicated. Any unreacted cysteines were alkylated with incubation with 5 mM IAA (either with or, in some runs, without 10 mM EDTA) for 2 hrs at 37°C. Previously oxidized cysteines were then reduced by treatment with 40 mM DTT for 30 min at 37°C. Newly reduced cysteines were alkylated by incubation with 85 mM N-ethyl maleimide (NEM) for 2 hrs at 37°C. The samples were separated on SDS-PAGE using Laemmli buffer containing 100 mM DTT and proteins visualized using Coomassie staining. Appropriate bands were excised and analyzed by liquid chromatography mass spectrometry (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 Shevchenko³⁶⁹. 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 2.6.2 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. For detection of dopamine metabolites on Parkin, the following variable modifications were included: 5,6-indolequinone (+C₈O₂NH₃, m/z shift +145), aminochrome (+C₈O₂NH₅, +147), aminochrome +2H (+C₈O₂NH₇, +149), and dopamine quinone (+C₈O₂NH₉, +151). These samples were prepared for analysis without any use of dithiothreitol or iodoacetamide. The observed spectra were matched against human sequences from SwissProt (version

2018-05) and also against an in-house database of common contaminants. The results were exported to Scaffold (Proteome Software, USA) for further validation and viewing. Analysis of the holoprotein and of three runs of H₂O₂-exposed r-parkin (Supplemental Table 2) were performed at the University of Western Ontario. There, samples were run on a QToF Ultima mass spectrometer (Waters) equipped with a Z-spray source and run in positive ion mode with an Agilent 1100 HPLC used for LC gradient delivery (University of Western Proteomics Facility).

MaxQuant analysis of mass spectrometry data

For applicable experiments, the raw MS data files were further processed with MaxQuant software version 1.6.5 and searched with the Andromeda search engine³⁴⁸. The reference fastas were set to uniprot-human (version 2019-02-12) and uniport-ecoli. The E. coli proteome was included to account for bacterial proteins present in the recombinant protein samples. The ‘second peptides’ and ‘match between runs’ settings were enabled. All other settings were left as default. Selected variable modifications included oxidation (Met), acetylation (protein N-terminus), and carbamidomethyl (Cys), as well as custom modifications for pyro-carbamidomethyl (N-terminal Cys), N-ethylmaleimide (Cys), and NEM+water (Cys). For data analysis, site-level intensity values were obtained from the MaxQuant-generated “CarbamidomethylSites” table which combines the intensity of MS1 signals from all peptides covering a particular cysteine residue.

Immunoprecipitation (IP) of brain parkin

Conjugation of anti-parkin antibody (Prk8, 808503, lot B209868) to magnetic beads at a final concentration of 10 mg of antibody/ mL of beads was done following the Magnetic Dynabeads Antibody Coupling Kit from Invitrogen (14311D). Human tissue lysates were also prepared using the “Sequential Extraction of Proteins from Tissue” protocol as described above with addition of 10 mM iodoacetamide prior to homogenization. TS tissue extracts (n=4) and SDS tissue extracts (n=8) were diluted in TS buffer, resulting in a final SDS concentration of 0.0175 % and 0.05 % respectively. For the IP, Prk8 conjugated agarose beads were first prepared by multiple washes with 1 mL of TS buffer using centrifugation (1000 x g at 4°C for 3 min) and adhesion to a strong magnet. Amounts of Prk8 conjugated agarose beads used for each experiment were approximated based on the amount of parkin (µg) / sample calculated by densitometry when the sample was compared to recombinant parkin protein standards using Western blotting with Prk8 primary antibody. The mixture was incubated for 16 h at 4°C with slow rotation. Unbound proteins, which did not bind to the Prk8 conjugated agarose beads, were separated from the beads by centrifugation (1000 x g at 4°C for 3 min) followed by adhesion to a strong magnet and saved as the IP “unbound” fraction. Beads from cellular or human IP were washed three times with 900 or 1000 µL respectively of ice-cold RIPA buffer (1 % nonionic polyoxyethylene-40, 0.1 % SDS, 50 mM Tris, 150 mM NaCl, 0.5 % sodium deoxycholate, 1 mM EDTA) using centrifugation (1000 x g at 4°C for 3 min) and adhesion to a strong magnet. Approximately 5- 10 µL of each wash was combined and saved as the IP “wash” fraction. To elute Prk8 bound proteins, 15-35 uL of 6X reducing Laemmli buffer (30 % SDS, 60 % glycerol, 0.3 % bromophenol blue, 0.375 M Tris, 100

mM DTT, pH 6.8) was added to the beads and the samples were boiled for 5 min. Following centrifugation (1000 x g at 4°C for 3 min), the supernatant was transferred to a fresh tube labeled “IP elute” and the beads were discarded. To assess IP efficiency, eluted fractions (IP elute), along with controls (input, unbound, wash and recombinant parkin protein standards) were run on SDS/PAGE and blotted with anti-parkin (MAB5512 or 2132S). Human IP elutes used in subsequent for mass spectrometry (MS) analysis were incubated with 500 mM N-ethyl maleimide (as indicated for select runs) for 16 h at 4°C prior to SDS-PAGE and further processing for MS (as described above). Gel slices corresponding to band sizes 50-75 kDa were excised and analyzed by LC-MS/MS.

***In vitro* melanin formation assay**

The recombinant protein samples were first prepared by exchanging the T200 protein buffer (50 mM Tris, 200 mM NaCl and 250 μ M TCEP, pH 7.5) for T200-TCEP (50 mM Tris and 200 mM NaCl, pH 7.5) using repeat centrifugations (8 times 4000 x g at 4°C for 10 min) in Amicon Ultra 10 kDa MWCO filters. The protein concentrations were measured and adjusted to 20 μ M using T200-TCEP. A 0.067 M sodium phosphate buffer, pH 6.0, was prepared by adding 33 mL of 0.1 M sodium phosphate buffer to 17 mL water and adjusting the pH using HCl. A stock solution of 100 mM dopamine HCl was prepared in 0.067 M sodium phosphate buffer and stock solutions of 100 mM reduced glutathione (GSH) and hydrogen peroxide were prepared in T200-TCEP.

Samples and controls were prepared in 100 μ L total volume and contained: 10 μ L of 20 μ M protein or T200-TCEP, 10 μ L of 100 mM dopamine or 0.067 M sodium phosphate buffer, 10 μ L of 100 mM glutathione or T200-TCEP buffer, and 70 μ L T200-TCEP. The

final concentration of protein was 2 μ M and the final concentration of reagents was all 10 mM. The samples and controls were plated in triplicate, and absorbance read at 405 and 475 nm every 90 sec for 1 h. The plate was then protected from light and incubated for 24 h. A final reading of absorbance at 405 and 475 nm was taken after 24 h.

Cell cytotoxicity assay

Human neuroblastoma cell line (M17 cells) wild-type, Vector (Myc), P5 (low stable expression of Myc-parkin) and P17 (high stable expression of Myc-parkin) were grown in 6 well culture plates, at 0.3×10^6 cell density (80% confluence) in Opti-MEM media (Gibco 11052-021) containing heat inactivated FBS (Gibco 10082-147), Pen/strep/Neo (5mg/5mg/10mg) (Gibco 15640-055), MEM non-essential amino acids (10mM) (Gibco 11140-050) and sodium pyruvate (100mM). For rescue experiments, flag-vector, flag-parkin, flag-p.G328E and flag-p.C431F pcDNA3.1+ were overexpressed in M17 wild-type cells. 4 μ g of cDNA was transfected using a 1:1 ratio of cDNA: Lipofectamine 2000 (52887, Invitrogen) in OPTI-MEM transfection medium. The cDNA and Lipofectamine 2000 was first incubated for 20 min at room temperature before being applied directly to the cells for 1 h at 37°C with 5 % CO₂ followed by direct addition of fresh growth medium. The cells were incubated another 24 hours at 37°C with 5 % CO₂.

Dopamine hydrochloride (Sigma H8502) 200 mM stock was prepared. The cells were washed with fresh media once and then incubated with media alone or supplemented with dopamine at final concentrations of 20 μ M and 200 μ M for 18-20 hours. Post dopamine stress, media was collected from all wells for cytotoxicity assay, the cells were harvested and lysed with TS buffer and centrifuged. The supernatant was collected and saved for

Western blot analysis and to assess total cell toxicity signal. The pellet was suspended in SDS buffer and centrifuged.

VybrantTM cytotoxicity assay kit (Molecular Probes V-23111) was used to monitor cell death through the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6yPD) from damaged cells into the surrounding medium. 50 µl of media alone (no cells), media from control and stressed cells and cell lysates were added to a 96-well microplate. Fifty µl of reaction mixture, containing reaction buffer, reaction mixture and resazurin, was added to all wells, and the microplate was incubated at 37°C for 30 mins. A microplate reader was used to measure either fluorescence with excitation at 560 nm and emission at 590 nm. A rise in fluorescence indicates a rise in G6PD levels i.e. a rise in cell death.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on paraffin-embedded sections and treated as previously described^{69,334,370}. Briefly, prior to antibody incubation, sections were deparaffinized in xylene and successively rehydrated through a series of decreasing ethanol concentration solutions. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol, followed by a standard citric acid-based antigen retrieval protocol to unmask epitopes. Sections were blocked in 10-20% serum in PBS-T to reduce non-specific signal. Sections were incubated overnight at 4°C in primary antibodies diluted in 1-5% serum in PBS-T according to the following concentrations: novel Parkin mAbs from Biolegend clones D (BioLegend, A15165D; 1:250), clone E (BioLegend, A15165E; 1:2000), and clone G (1:250), PRK8 (BioLegend, MAB5512; 1:500), Lamp3

(Santa Cruz, SC5275; 1:100), LC3B (Sigma, L7543-200uL; 1:100), VDAC (MitoScience, MSA03; 1:100). Biotinylated secondary antibodies (biotinylated anti mouse IgG (H+L) made in goat; Vector Labs, BA-9200, biotinylated anti-rabbit IgG (H+L) made in goat; Vector Labs, BA-1000) were diluted to 1:225 and sections were incubated for 2 hours at room temperature. The signal was amplified with VECTASTAIN® Elite® ABC HRP Kit (Vector Labs, PK-6100), and visualized via standard DAB solution, 55mM DAB, or Vina green (Biocare Medical, BRR807AH), or most commonly metal enhanced DAB (Sigma, SIGMAFAST™ DAB with Metal Enhancer D0426). Samples were counterstained with Harris Modified Hematoxylin nuclei stain and dehydrated through a series of increasing ethanol concentration solutions and xylene. Permount (Fisher Scientific, SP15-100) was used for mounting and slides were visualized with high magnification images via a Quorum Slide Scanner (Ottawa Hospital Research Institute).

Immunofluorescence (IF) and confocal microscopy

Paraffin-embedded human midbrain sections were stained by routine indirect immunofluorescence with the following details. Antigen retrieval was performed in Tris-EDTA buffer pH 9 for 10 mins. Primary antibodies were incubated overnight at 4C. Details for primary antibodies anti-Parkin Clone E (1:500), anti-LAMP3 (1:250) are described above. A 40 minute incubation with the following secondary antibodies was performed: goat anti-mouse alexa fluor 488 (1:200), goat anti-rabbit alexa fluor 594 (1:500). Slides were mounted with fluorescence mounting medium with DAPI. Stained

sections were imaged using a Zeiss LSM 880 AxioObserver Z1 with an Airyscan Confocal Microscope then processed and analyzed using Zeiss Zen and Fiji software.

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Differences between two groups were assessed using an unpaired t-test. Differences among 3 or more groups were assessed using a one-way or two-way ANOVA followed by Tukey's post hoc corrections to identify statistical significance. Subsequent post hoc tests are depicted graphically and show significance between treatments. For all statistical analysis a cut-off for significance was set at 0.05. Data is displayed with p values represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Logistic/linear regression modelling: Linear regression (for continuous dependent variable, e.g. H_2O_2 level, mRNA level) or logistic regression (for binary dependent variable, e.g., parkin present in TS fraction) modelling were performed. Furthermore, to address the effect of age on parkin solubility, receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) were calculated, as reported³⁶⁹.

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L.W., J.K., A.C.N., Q.J., R.S., J.L., M.Z., K.R.B., A.T., X.D., L.P., G.T. performed experiments; and C.R.S., A.B.W., E.T., A.H., A.P., J.A.C., provided data, tissue specimens and critical comments. *Analysis:* J.M.T., D.N.E.K., J.L., T.K.F., G.S.S., L.P., G.T., J.M.W., P.T., J.J.T., M.G.S. performed data analyses. *Study supervision:* P.T., J.J.T., M.G.S. *Overall responsibility:* M.G.S. This work is dedicated to the memories of Mr. Bruce Hayter (1962-2019), a tireless advocate for persons with young-onset parkinsonism, and our colleague, Dr. Arne Holmgren (1940-2020), a pioneer in redox biology.

Competing interests: Drs. B. O’Nuallain, M. Jin, L. Wang, P. Taylor are (or were) employees of BioLegend Inc. (Dedham, MA., USA). The Ottawa Hospital receives payments from BioLegend Inc. related to licensing agreements for immunological reagents related to parkin and α -synuclein. Dr. M. Schlossmacher received travel reimbursements from the Michael J. Fox Foundation for Parkinson’s Research for participation in industry summits and consulting fees as well as royalties from Genzyme-Sanofi for patents unrelated to this work. Dr. G. Toth is an employee and a shareholder of Cantabio Pharmaceuticals. Dr. A. Holmgren (deceased) served as chairman and senior scientist at IMCO Corporation Ltd AB, Stockholm, Sweden. No additional, potentially competing financial interests are declared.

Additional Information:

Data and materials availability: Original data associated with this study are available in the main text and extended data figures and tables; additional data will be made available upon request.

Supplementary Information is available for this manuscript.

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Supplementary Table 2.1: Summary of human brain tissue specimens used in this study with designated small letter identification, as used in the Figures. Abbreviations used: F ctx, frontal cortex; PMI, *post mortem* interval; hrs, hours; HCO, healthy control; NCO, neurological control; PSM, parkinsonism, n.d., not documented. For parkin solubility: 1, present in Tris-saline (TS) buffer; 0, not present in TS.

ID in figures	Sample ID	Brain region	Age	Sex	PMI (hrs)	Diagnosis	Parkin Solubility	H ₂ O ₂ /Tissue ratio	Appears in figures
a	1	Frontal cortex	5	F	33	HCO	1		1b, 1d, 1f, 1g, 1h, E1a, E1b, E2c
b	2	Frontal cortex	5	F	20	HCO	1	2.551	1b, 1d, 1f, 1g, 1h, 1j, 1k, 1l, 1m, E1a, E1b, E2c
	3	Frontal cortex	8	M	5	HCO	0	1.780	1b, 1f, 1g, 1l, 1m, E2c
t	4	Frontal cortex	13	M	13	HCO	1	2.217	1b, 1f, 1g, 1j, 1k, 1l, 1m, E2c
	5	Frontal cortex	15	F	9	HCO	1	1.349	1b, 1f, 1g, 1l, 1m, E2c
j	6	Frontal cortex	16	F	20	HCO	1		1b, 1f, 1g, 1h, E1b, E2c
c	7	Frontal cortex	16	F	14	HCO	1	1.016	1b, 1d, 1f, 1g, 1l, 1m, E1a, E2c
ff	8	Frontal cortex	17	M	23	HCO	0	0.701	1b, 1f, 1g, 1l, 1m, 2g, E2c
	9	Frontal cortex	17	M	22	HCO	1		1b, 1f, 1g, E2c
k	10	Frontal cortex	20	F	19	HCO	1		1b, 1f, 1g, E2c
o	11	Frontal cortex	20	M	8	HCO	1		1b, 1f, 1g, E2c, E2e
	12	Frontal cortex	20	M	6	HCO	0		1b, 1f, 1g, E2c
	13	Frontal cortex	20	M	5	HCO	1		1b, 1f, 1g, E2c
	14	Frontal cortex	21	M	30	HCO	1		1b, 1f, 1g, E2c
	15	Frontal cortex	28	M	33	NCO	1		1b, 1f, 1g, E2c
d	16	Frontal cortex	29	F	18	NCO	0	3.061	1b, 1d, 1f, 1g, 1l, 1m, E1a, E2c
	17	Frontal cortex	30	M	20	HCO	0	6.089	1b, 1f, 1g, 1l, 1m, E2c
	18	Frontal cortex	36	M	20	HCO	0	5.665	1b, 1f, 1g, 1l, 1m, E2c
u	19	Frontal cortex	37	F	13	HCO	0		1b, 1f, 1g, 1j, 1k, 2g, E2c
v	20	Frontal cortex	38	M	17	HCO	0		1b, 1f, 1g, 1j, 1k, E2c
	21	Frontal cortex	39	M	23	HCO	1		1b, 1f, 1g, E2c
e	22	Frontal cortex	39	M	14	HCO	1		1b, 1d, 1f, 1g, E1a, E2c
	23	Frontal cortex	42	M	18	HCO	1		1b, 1f, 1g, E2c
f	24	Frontal cortex	43	F	22	HCO	0	4.601	1b, 1d, 1f, 1g, 1l, 1m, E1a, E2c
	25	Frontal cortex	44	F	21	NCO	0		1b, 1d, 1f, 1g, E1c, E2c
	26	Frontal cortex	49	F	16	HCO	0	5.622	1b, 1f, 1g, 1l, 1m, E2c
s	27	Frontal cortex	49	F	14	HCO	0		1b, 1f, 1g, E2c
m	28	Frontal cortex	54	M	16	NCO	0		1b, 1f, 1g, E2c
	29	Frontal cortex	54	F	23	NCO	1		1b, 1f, 1g, E2c
w	30	Frontal cortex	55	F	16	HCO	0	5.829	1b, 1f, 1g, 1l, 1m, E2c
q	31	Frontal cortex	56	M	23	HCO			1b, 1f, 1g, 1h, E1b, E2c
	32	Frontal cortex	57	M		HCO	0		1b, 1f, 1g, E2c
g	33	Frontal cortex	62	M	15	NCO	0	6.525	1b, 1d, 1f, 1g, 1j, 1k, 1l, 1m, E1a, E2c
x	34	Frontal cortex	65	M	5	PSM	0	6.473	1b, 1f, 1g, 1j, 1k, 1l, 1m, E2c
z	35	Frontal cortex	65	M	14	PSM	0	9.112	1b, 1f, 1g, 1j, 1k, 1l, 1m, E2c
n	36	Frontal cortex	65	F	42	HCO	0	5.768	1a, 1b, 1f, 1g, 1l, 1m, E2c, E2e
h	37	Frontal cortex	66	M		HCO	0	6.674	1b, 1d, 1f, 1g, 1l, 1m, E1a, E2c
p	38	Frontal cortex	68	M	17	HCO	0	2.514	1b, 1f, 1g, 1h, 1l, 1m, E1b, E2c
i	39	Frontal cortex	70	F		HCO	0	6.897	1b, 1d, 1f, 1g, 1l, 1m, E1a, E2c
r	40	Frontal cortex	70	M		HCO	0	5.459	1b, 1f, 1g, 1h, 1l, 1m, E1b, E2c
aa	41	Frontal cortex	72	M		PSM	1	6.274	1b, 1f, 1g, 1j, 1k, 1l, 1m, E2c
	42	Frontal cortex	75	M	48	HCO	1	2.878	1b, 1d, 1f, 1g, 1l, 1m, E2c
	43	Frontal cortex	75	F	13	NCO	0		1b, 1f, 1g, E2c
y	44	Frontal cortex	75	M	17	PSM	0		1b, 1f, 1g, 1j, 1k, E2c
	45	Frontal cortex	76	M	74	PSM	0		1b, 1f, 1g, E2c
l	46	Frontal cortex	85	F	15	NCO	0		1b, 1f, 1g, 1j, 1k, E2c
bb	47	Midbrain	26	M	2	NCO	0		1c, E2b
	25	Midbrain	44	F	21	HCO	0		1c
ee	48	Midbrain	44	F	5	NCO	1		1c, E2b
	49	Midbrain	45	M	13	NCO	0		1c
	50	Midbrain	47	F	20	NCO	0		1c
	51	Midbrain	56	M	44	PSM	0		1c
	52	Midbrain	60	M	16	PSM	0		1c
	53	Midbrain	61	M	20	NCO	1		1c
cc	54	Midbrain	61	M	3.5	NCO	0		1c
n	36	Midbrain	65	F	42	HCO	0		1c, E2a
dd	55	Midbrain	65	M	6	NCO	0		1c
	41	Midbrain	72	M		PSM	1		1c
	56	Midbrain	74	F		NCO	0		1c
	42	Midbrain	75	M	48	HCO	0		1c
	57	Midbrain	75	M	70	PSM	0		1c
	45	Midbrain	76	M	74	PSM	0		1c
	58	Midbrain	79	M		PSM	0		1c
	59	Midbrain	82	M	48	PSM	1		1c
SC-1	60	Spinal cord/muscle	68	F	2.5	NCO	1		1i, E1e
SC-2	61	Spinal cord/muscle	64	F	2	HCO	1		1i
SC-3	62	Spinal cord/muscle	71		2		1		1i
SC-4	63	Spinal cord/muscle	50	M	4		1		1i

Supplementary Table 2.2: Parkin's cysteine residues are redox active.

Aliquots of human recombinant (r-) parkin that were oxidized by variable concentrations of H₂O₂ vs. control preparations were differentially labelled with iodoacetamide (IAA) and/or N-ethylmaleimide (NEM; as in Figure 4A) to identify reduced cysteines (IAA) or reversibly-oxidized residues (NEM). Proteins were subjected to LC-MS/MS and analyzed using Mascot Scaffold PTM to identify IAA (•) or NEM (+) adducts indicating when these were detectable on individual residues. Cysteines that were not detected as modified in individual runs are also listed (n/d). Note that cysteines within all four RING domains of parkin as well as in the linker and UbL domains can be variably modified. Corresponding data can be found in Extended Data Fig. 4h. For comparison, see **Extended Data Table 1** that lists modified cysteines identified in parkin purified from human brain.

Treatment		Control	Control	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂
Run		IAA+NEM	IAA	20μM	1mM	4.5mM	4.5mM	4.5mM
Region	Cysteine Residue							
UBL	59		•	n/d	•	• +	•	•
Linker	95	•	•	•	•	• +	• +	• +
RING0	150	•	•	•	•	• +	• +	•
	154	•	•	n/d	•	• +	• +	•
	166	n/d	n/d	•	• +	n/d	•	•
	169	n/d	•	•	• +	n/d	•	•
	182	•	•	•	n/d	• +	• +	•
	196	•	•	•	•	• +	• +	• +
	201	•	•	•	•	• +	• +	• +
	212	•	•	•	n/d	• +	• +	• +
RING1	238	•	•	• +	•	• +	•	• +
	241	•	•	• +	•	• +	•	•
	253	•	•	• +	•	• +	•	•
	260	•	•	n/d	n/d	• +	•	•
	263	•	•	n/d	n/d	• +	•	•
	268	•	•	n/d	n/d	• +	•	•
	289	•	•	n/d	n/d	• +	• +	• +
	293	•	•	n/d	•	•	• +	• +
	323	•	•	n/d	n/d	• +	• +	• +
	332	•	•	n/d	n/d	• +	•	•
IBR	337	•	•	n/d	•	• +	•	• +
	352	•	•	•	•	•	• +	• +
	360	•	•	•	•	• +	•	•
	365	•	•	•	n/d	• +	•	•
	368	•	•	n/d	n/d	• +	• +	•
	377	•	•	•	n/d	• +	• +	• +
	418	n/d	n/d	n/d	•	n/d	n/d	n/d
RING2	421	n/d	n/d	n/d	• +	n/d	• +	• +
	431	n/d	•	n/d	•	n/d	•	•
	436	n/d	n/d	n/d	• +	n/d	•	•
	441	n/d	n/d	n/d	• +	n/d	•	•
	446	•	•	n/d	n/d	• +	• +	• +
	449	•	•	n/d	n/d	• +	• +	•
	451	•	•	n/d	n/d	• +	•	•
	457	•	•	•	•	• +	•	• +

• IAA + NEM

**Chapter 3: Manuscript 2 - Parkinson Disease-Linked Parkin
Mediates Redox Reactions That Lower Oxidative Stress in
Mammalian Brain**

Statement of author contribution

This manuscript, a follow-up to chapter 2, further validates parkin's function in mediating redox reactions, maintaining redox homeostasis, and lowering chronic oxidative stress in mammalian brains. Here, we utilize *in vitro* and *in vivo* experiments, using both cellular and animal models to investigate parkin's cysteine-mediated oxidation and insolubility, in exchange to decreasing the levels of markers of oxidative stress, in cells, and mouse and human tissues. Additionally, we establish a feedback loop between glutathione metabolism and parkin, whereby reduced glutathione levels are increased in the absence of parkin, due in part to parkin's ability to recycle oxidized glutathione through S-glutathionylation.

Daniel El Kodsi established the majority of the experimental design, in close collaboration with Dr. Jackie Tokarew, specifically in regards to optimizing assays (incubation times, concentrations, reagents, buffers etc.), and discussing published literature to ensure sound scientific methodology. **Daniel El Kodsi** performed all cellular experiments, including parkin oxidation and insolubility, ROS measurements, and cell death assays, in HEK293 and CHO cells; with the exception of **Extended Data Figure 3.2i**, which was performed by Dr. Jackie Tokarew. **Daniel El Kodsi** was in charge of all mouse colony breeding and maintenance, and tissue (brains and hearts) harvest and dissection. **Daniel El Kodsi** conducted all the mouse-related experiments, including H₂O₂-dependent parkin oxidation and insolubility; mitochondria and cytosol fractionation; ROS measurements; acute MPTP treatment (with the help of Dr. Doo Soon Im); immunoprecipitation and mass spectrometry (with the help of Dr. Andy Ng and Dr. Lawrence Puente); and the development and characterization of the bi-genic mouse model (initially started by Dr. Tohru Kitada; further

characterized in manuscript 3). **Daniel El Kodsi** measured the levels of oxidative stress markers, including carbonyl content and nitrotyrosination, with the exception in human brains in **Figure 3.2h**, which was performed by Nathalie Lengacher. Dr. Jackie Tokarew provided the recombinant proteins used in the *in vitro* assays, which were performed by **Daniel El Kodsi**, including r-parkin, DJ-1 and α -synuclein. **Daniel El Kodsi** carried out all reduced and oxidized glutathione measurements in cells and mouse and human brains (with the help of Dr. Chantal Peliggi), with the exception of **Figure 3.3e** and **Figure 3.4a**, which were carried out by Dr. Jackie Tokarew. Dr. Jackie Tokarew and Dr. Rajib Sengupta were in charge of all parkin glutathionylation experiments in **Figure 3.4** and **Extended Data Figure 3.4**. Heather Boston performed the mitochondrial enzymatic activity assays (**Figure 3.2j** and **Extended Data Figure 3.3e-g**); Nathalie Lengacher carried out the human brain fractionation experiment (**Extended Data Figure 3.3a**); Dr. Bojan Shutinoski conducted the mRNA quantification (**Extended Data Figure 3.4e**; **Daniel El Kodsi** designed the primers and provided the tissues); and Dr. Juan Li was in charge of the linear regression modeling (**Figure 3.2i** and **Figure 3.3g**). Lastly, the initial draft of the manuscript was written by **Daniel El Kodsi**, including the current materials and methods section and referencing.

Parkinson Disease-Linked Parkin Mediates Redox Reactions That Lower Oxidative Stress in Mammalian Brain

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Abstract

We recently hypothesized that parkin contributes to redox homeostasis in human brain and provided *ex vivo* evidence. Here, we examined its anti-oxidant activities *in vivo*. We demonstrate that elevated oxidative stress promoted parkin insolubility in mice. In adult brain parkin was post-translationally modified including at cysteines 195 and 252, which was augmented by oxidative stress. Under basal conditions hydrogen peroxide (H_2O_2) concentrations were unchanged in *prkn*^{-/-} brain. However, parkin-dependent lowering of cytosolic H_2O_2 was observed when mitochondria were impaired due to neurotoxicant exposure or *Sod2* haploinsufficiency. Intriguingly, protein carbonylation and nitrotyrosination were elevated in the cytosol but not in mitochondria of *prkn*^{-/-} mice. Nevertheless, elevated oxidative stress in the cytosol altered mitochondrial enzyme activities due to thiol network changes. In parkin's absence reduced glutathione (GSH) concentrations were increased including in human cortex. This compensation was attributed to elevated oxidized glutathione (GSSG)-reductase activity. We also found that human parkin directly recycled GSSG to GSH, which S-glutathionylated parkin at cysteines 59 and 95. Our results demonstrate that cytosolic parkin mediates anti-oxidant reactions including H_2O_2 reduction and glutathione recycling. These activities lead to a range of oxidative modifications in parkin. In parkin-deficient brain oxidative stress rises despite compensatory efforts to maintain redox balance.

Introduction

Parkinson disease (PD) is a progressive, heterogeneous disorder of the human brain that remains incurable. Young-onset, autosomal-recessive PD (ARPD) due to deficiency in parkin (encoded by *PRKN*) is characterized by the degeneration of dopamine producing neurons in the *S. nigra* and *L. coeruleus*¹⁹⁹. In the ageing human brain, these neurons are susceptible to degeneration because of unique features. These include: extensive arborization; a high number of axonal mitochondria; the presence of metals in redox-reactive forms³⁷¹; ongoing generation of toxic dopamine metabolites in the cytosol; a greater need to buffer Ca^{2+} ions; and collectively, a greater degree of oxidative stress^{51,356,372}.

Parkin is a RING-carrying protein with ubiquitin ligase activity that is involved in the regulation of mitophagy and immune-related functions^{323,268,150}. Their contributions to ARPD development remain unknown. We recently described that parkin itself is highly oxidized in human brain and discovered that it acts as a redox molecule to decrease dopamine metabolism-associated stress^{260,373}.

Oxidative stress and mitochondrial damage have been implicated in the pathogenesis of several brain disorders including PD⁸⁴. Mitochondrial dysfunction, as induced by the neurotoxicants 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, augments oxidative stress³⁷⁴. The integrity of the cellular thiol pool, a network formed by glutathione and the cysteine proteome, is essential in maintaining redox homeostasis in long-lived neurons. The reduced form of glutathione (GSH) is critical as an anti-oxidant.

Accordingly, a decline in GSH has been implicated in many human disorders including neurodegenerative diseases³⁴³.

Genomic deficiency in parkin in mice does not induce loss of dopamine neurons, and *prkn*^{-/-} animals do not develop parkinsonism. However, unbiased studies have revealed proteomic changes in *prkn*^{-/-} brains^{94,95}. There, we noted that a majority of dysregulated proteins had been previously identified as enzymes (55/92; 59.8%); many were also known as redox-sensitive proteins (72/92; 78.2%), *e.g.*, glyoxalase and aconitase-2^{94,95}. These findings suggested to us that posttranslational modifications, rather than protein abundance, could have explained their detection.

When taken together with parkin's own sensitivity to pro-oxidants^{260,333}, we hypothesized that: one, thiol-rich parkin is altered during cellular redox changes; two, parkin plays an active role in lowering oxidative stress *in vivo*, thus influencing redox-sensitive enzymes; and three, the loss of parkin's anti-oxidant effects contributes to mitochondrial change. We therefore characterized parkin's redox activity *in vitro*, explored redox changes in cells and mouse models with diminished parkin expression, and investigated its effects on the thiol network. This work was informed by parallel efforts, which focused on parkin's processing in human midbrain and dopamine metabolism³⁷³.

Results

Mitochondrial and cytosolic stress lead to oxidation of cellular parkin

We first analyzed parkin's response to cytosolic and mitochondrial stressors. Chinese hamster ovary cells stably expressing wild-type (WT) human *PRKN* cDNA (CHO-parkin) and control cells, which showed little endogenous parkin by Western blotting, were exposed to different levels of hydrogen peroxide (H_2O_2 , 0.2 or 2mM) or carbonyl-cyanide-m-chlorophenyl-hydrazine (CCCP, 10mM). Under these stress conditions, which did not cause cell death, we observed a time-dependent reduction in parkin's monomer (~52.5 kDa) and reciprocal increase in high molecular weight (HMW) species (**Fig. 3.1a**)³³³. These changes were seen under non-reducing electrophoresis conditions and interpreted as involving thiol-based oxidation. Intriguingly, this oxidation was specific to parkin when compared with endogenous, redox-sensitive and ARPD-linked Dj-1³⁷⁵ (**Fig. 3.1a**).

In stress-exposed, human embryonic kidney (HEK293) cells transiently over-expressing *PRKN*, we also observed HMW smear formation. Parkin became progressively gel excluded (aggregated) and insoluble when exposed to increasing H_2O_2 concentrations (**Fig. 3.1b**). Because these HMW parkin species were reversed with dithiothreitol (DTT; **Extended Data Fig. 3.1a,c**), we postulated that they occurred due to oxidation of parkin's cysteines³³³. We tested this using N-ethylmaleimide (NEM) and iodoacetamide (IAA), which irreversibly alkylate free thiols. Pre-incubation of cells with NEM or IAA blocked HMW formation of parkin and preserved its solubility during H_2O_2 stress (**Fig. 3.1b; Extended Data Fig. 3.1b**). Complementing these findings, we mapped several

cysteines in recombinant (r-) parkin as oxidized when nascent protein was subjected to rising H₂O₂ concentrations *in vitro*, which led to its aggregation and insolubility³⁷³. Intriguingly, exposure to DTT also lowered parkin solubility in cells (**Extended Data Fig. 3.1c**), which suggested that parkin's structure is altered under excessive redox conditions. These results demonstrated that human parkin undergoes thiol chemistry-based changes in cells.

Excessive ROS exposure promotes parkin insolubility in mice

Under stress-free conditions, parkin resides in the cytosol of cells (**Fig. 3.1a,b**) and rodent brain^{299,332}. In contrast, in human control brain parkin is largely insoluble after the 4th decade of life^{332,373}. To better understand the link between parkin's oxidation and its insolubility, we sought to validate these findings in mice. First, freshly dissected brains from WT mice were homogenized either in the presence or absence of H₂O₂ to generate additional reactive oxygen species (ROS). Lysates were then fractionated and separated into soluble *vs.* insoluble fractions. Exposure to H₂O₂ *in situ* led to a significant loss in solubility of mouse parkin and recovery in the insoluble fraction (P<0.001), as monitored by reducing SDS/PAGE. No such shift was seen for murine Dj-1 (**Fig. 3.1c,d**).

Secondly, we examined parkin's response to oxidative stress generated *in vivo*. We injected a single dose of MPTP into the peritoneum of adult mice at a dose that induces oxidative stress without neuronal death³⁷⁶. After one hour, brains were collected and serially fractionated. The exposure to MPTP led to a decline of >50% in soluble parkin

when compared to saline-treated controls, and parkin's detection rose in the insoluble fraction ($P < 0.05$; **Fig. 3.1e**; **Extended Data Fig. 3.1d,e**).

In a third approach, we tested whether a genetic cause of higher ROS production altered parkin solubility. We chose *Sod2*^{+/-} haploinsufficient mice with lower expression of mitochondrial superoxide dismutase-2 (MnSOD). Reduced MnSOD activity (**Extended Data Fig. 3.1h,i**) results in elevated oxidative stress³⁷⁷. In adult *Sod2*^{+/-} brain, parkin solubility also shifted ($P < 0.05$; **Fig. 3.1f**; **Extended Data Fig. 3.1j,k**). We concluded that rising oxidative stress levels promote parkin insolubility in mouse brain. We postulated that this change correlated with more oxidized thiols within parkin.

MPTP exposure increases parkin oxidation in the brain

To test this, we employed liquid chromatography and mass spectrometry (LC-MS/MS). Brains from mice injected with MPTP or saline were homogenized in the presence of IAA to alkylate free cysteines and prevent their oxidation during processing. Parkin-enriched isolates were separated by SDS/PAGE (**Extended Data Fig. 3.1f**). Monomeric, alkylated parkin was gel-excised, trypsin digested and subjected to LC-MS/MS. We recorded a <20% decline in the relative amount of unmodified cysteines in parkin peptides from MPTP-treated mice compared to those from saline-injected controls (**Extended Data Table 3.1**). Furthermore, we found that several cysteines had been oxidized under normal conditions, *e.g.*, to dehydroalanine, sulfinic and sulfonic acid (**Extended Data Table 3.1**). Intriguingly, residues C195 and C252 were frequently identified as irreversibly oxidized in mouse brain (**Extended Data Table 3.1**; **Extended**

Data Fig. 3.1g). In studies of human parkin, we found that oxidation of orthologous cysteines, *e.g.*, C253, contributed to its progressive insolubility³⁷³.

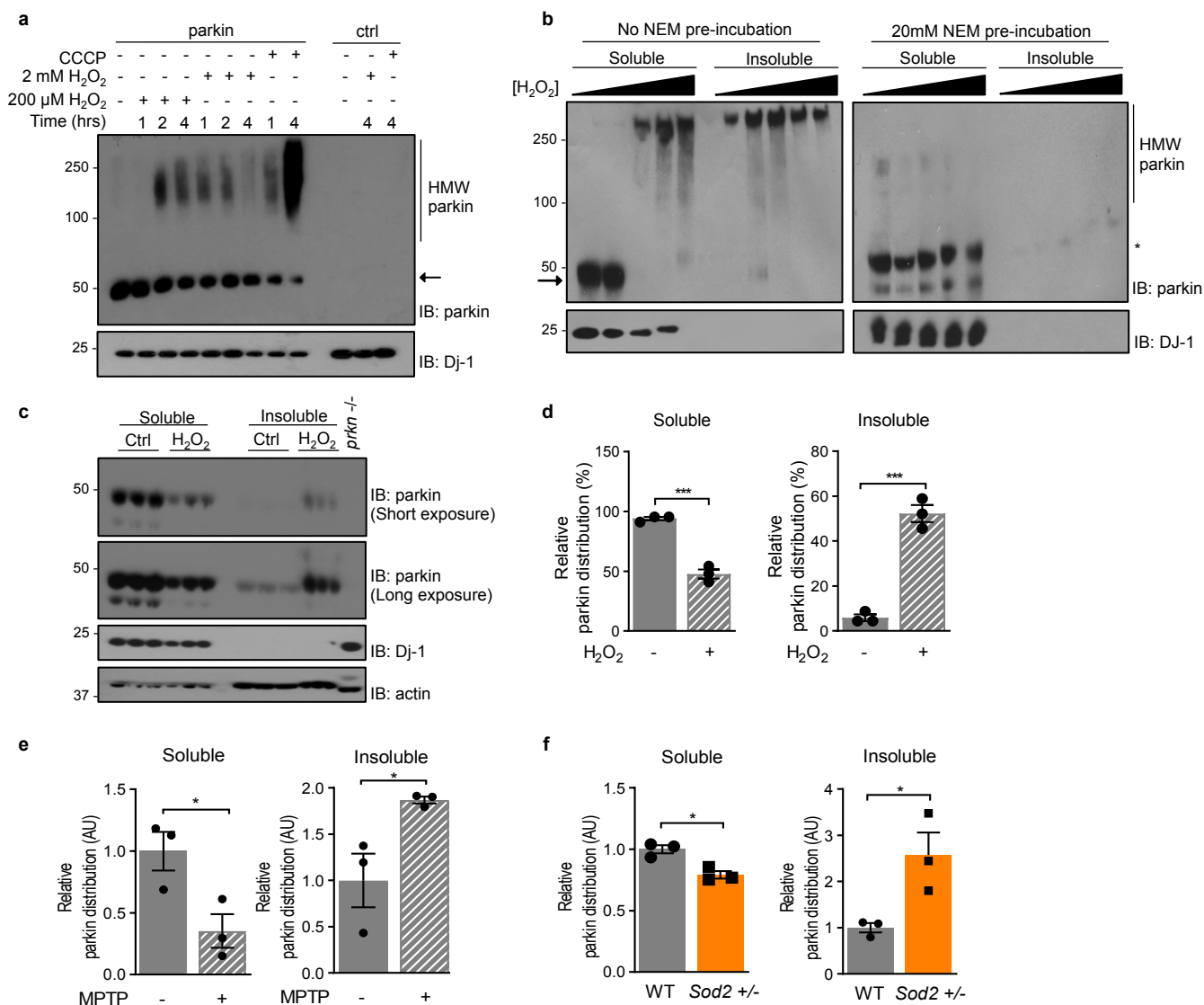
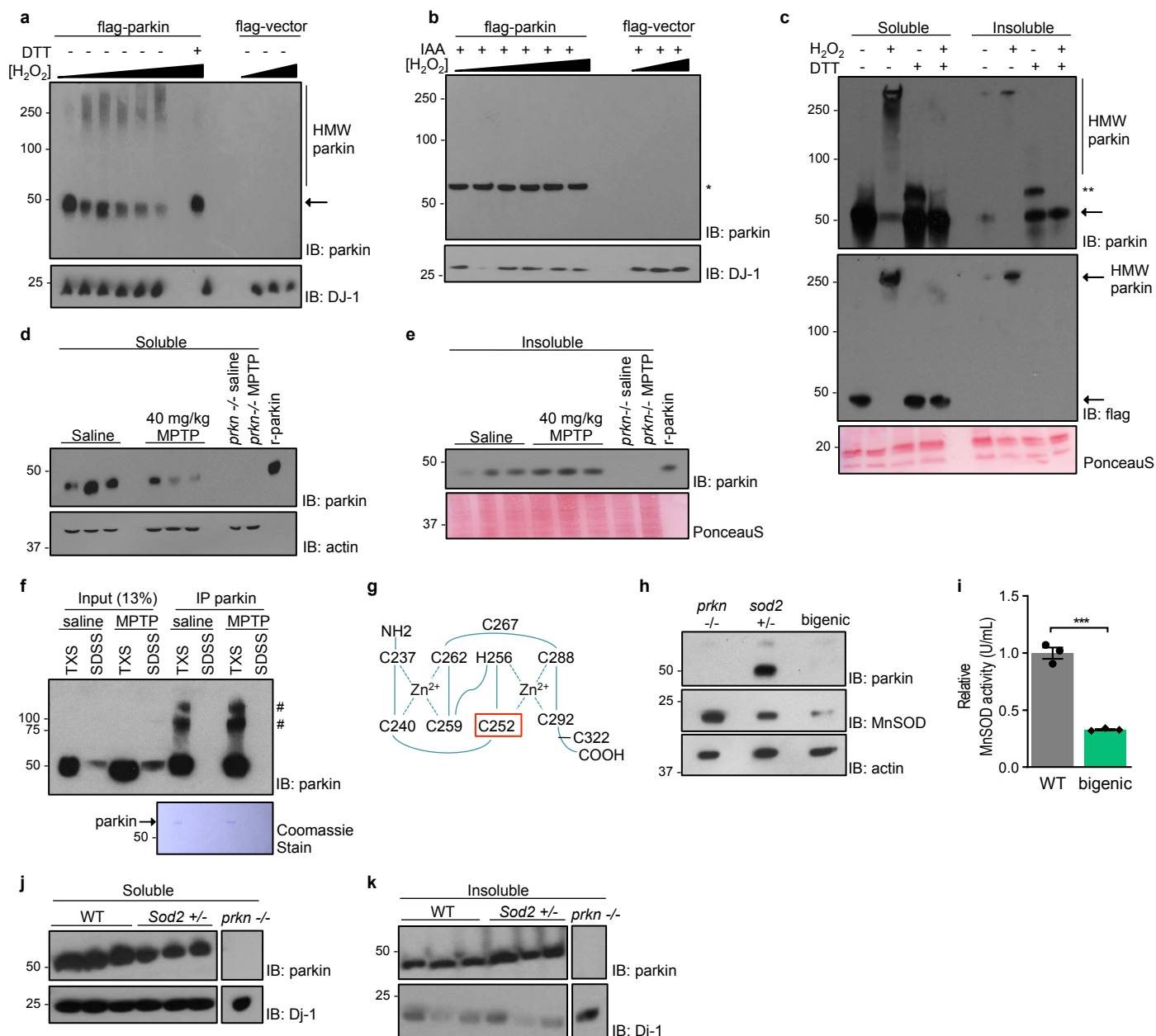


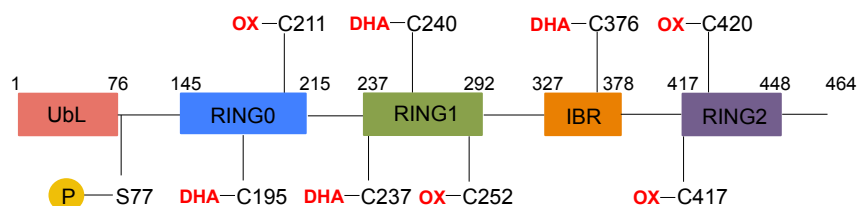
Figure 3.1: Mitochondrial and cytosolic stressors lead to oxidation of cellular parkin promoting its insolubility. Representative Western blots of parkin and Dj-1 distribution in, **a**, CHO-parkin and control cells under oxidizing conditions (200μM and 2mM H₂O₂; and 10μM CCCP), **b**, soluble and insoluble fractions from HEK293-parkin and control cells incubated with increasing concentrations of H₂O₂ (2μM to 2M) with or without 20mM n-ethylmaleimide (NEM) pre-incubation, and **c**, soluble and insoluble fraction from 2-4 mths-old wild-type C57Bl/6 mouse brains incubated with saline or 1% H₂O₂. (←) Monomeric parkin (*) Alkylated-parkin. Quantification of parkin's signal distribution in brain homogenates from, **d**, 2-4 mths-old wild-type C57Bl/6 mouse brains incubated with saline or 1% H₂O₂, **e**, 2-4 mths-old wild-type C57Bl/6 mice treated with a 40mg/kg intraperitoneal injection of MPTP toxin or saline. **f**, 6-8 mths-old C57Bl/6 wild-type (WT) and *Sod2*^{+/-} mouse brains (soluble and insoluble fractions are shown). A Student t-test was used for statistical analysis (* = < 0.05, *** = < 0.001).



Extended Data Figure 3.1: Mitochondrial and cytosolic stressors lead to oxidation of cellular parkin promoting its insolubility.

Representative Western blots of parkin and DJ-1 in HEK293-parkin or control cells incubated with increasing concentrations of H₂O₂ (2μM to 20mM) **a**, with the addition of dithiothreitol (DTT) (100mM, 30 mins; lane 8) or **b**, pre-incubating with iodoacetamide (IAA) (10mM, 15 mins). **c**, Representative Western blots of parkin and flag in cell lysates from HEK293-parkin or controls cells under oxidizing and/or reducing conditions. (←) Monomeric parkin, (*) Alkylated-parkin and (**) DTT-modified-parkin. Representative Western blots of parkin distribution in **d**, soluble, and **e**, insoluble fractions of whole or hemi brain homogenates from mice treated with saline or MPTP. **f**, Representative Western blots of immunoprecipitated parkin from mouse brains treated with saline or MPTP. (#) IgG related bands. **g**, Schematic representation of parkin's RING1 domain with the position of an oxidized cysteine residue highlighted (C252). **h**, Representative Western blots of parkin and MnSOD in brains of 2-4 mths-old *prkn*^{-/-}, *Sod2*^{+/-} and bi-genic mice. **i**, MnSOD activity in brains of 2-4 mths-old WT and bi-genic mice. Representative Western blots of parkin and DJ-1 distribution in **j**, soluble, and **k**, insoluble fractions of brains from WT and *Sod2*^{+/-} mice. A Student t-test was used for statistical analysis (***) (= < 0.001).

Run	1		2		Mean	
Treatment	Saline (n=1)	MPTP (n=1)	Saline (n=2)	MPTP (n=2)	Saline	MPTP
Number of unique parkin peptides identified	11	18	11	27	11.0	22.5
Number of amino acids identified	106	154	123	323	114.5	238.5
% coverage	23	33	27	70	25.0	51.5
Number of cysteines identified	6	10	7	26	6.5	18.0
% of total cysteines	17.6	29.4	20.6	76.5	19.1	52.9
Number of IAA-modified cysteines identified	6	8	7	22	6.5	15.0
Ratio IAA-cysteines/total cysteines identified	100.0	80.0	100.0	84.6	100.0	82.3
Cysteine -> Dehydroalanine	C195	C195	C195, C240, C252	C195, C237, C240, C252, C376		
Sulfonic acid (Cys trioxidation)	C252	C252	C252, C417, C420	C211, C252		
Sulfinic acid (Cys dioxidation)	n/d	n/d	n/d	C252		
Phosphorylation	n/d	n/d	n/d	S77		
Methionine oxidation	M191, M457	M191, M457	M191, M457	M191, M326, M457		



Extended Data Table 3.1: Parkin cysteines are oxidized in adult mouse brain.

Table summarizing mass spectrometry results highlighting the ratios of the number of IAA-tagged and free cysteines divided by the total number of identified cysteines in wild-type parkin enriched from the brains of mice treated with MPTP (2 independent runs, n=3 brains each) compared to saline controls (2 independent runs, n=3 brains each) under reducing and alkylating conditions (see Methods for details). The means of both runs in each treatment are shown with the ratio (in percent) of IAA-tagged cysteines to total identified cysteines highlighted. Select, oxidative stress-induced, posttranslational modifications found at murine parkin cysteines are highlighted in the table (bottom), *e.g.*: cysteine to dehydroalanine; cysteine dioxidation (=sulfinic acid); and cysteine trioxidation (=sulfonic acid). Phosphorylation and methionine oxidation events (table only) are also listed. Graphic summary of murine parkin domains with selected residues for identified PTMs. OX, oxidized cysteines (sulfinic or sulfonic acid); DHA, dehydroalanine; P, phosphorylation.

Parkin reduces hydrogen peroxide in a thiol-dependent manner

We recently provided evidence that WT r-parkin reduced H_2O_2 to water *in vitro* by reciprocal oxidation of its thiol groups³⁷³; ARPD-linked point mutants did so less well. Here, we further explored the mechanisms underlying this activity. Human r-parkin was ~5-fold more effective in lowering H_2O_2 levels than GSH at equimolar concentrations, but was less potent than catalase (**Extended Data Fig. 3.2a**). We found that this activity was free thiol-based, because r-parkin's ability to reduce H_2O_2 was abrogated when its cysteines were either oxidized prior to the reaction or irreversibly blocked by NEM (**Extended Data Fig. 3.2b,c**). Parkin did not exhibit peroxidase-type activity (**Extended Data Fig. 3.2d**).

Other PD-linked proteins, *e.g.*, α -synuclein and DJ-1 (**Extended Data Fig. 3.2e**), had no detectable H_2O_2 -lowering activity (**Fig. 3.2a**). Bovine serum albumin with the same number of cysteines as human parkin (35) and two RING-carrying E3 ubiquitin ligases, *e.g.*, RNF43 and HOIP^{cd} (**Extended Data Fig. 3.2e,f**), also failed to lower H_2O_2 (**Fig. 3.2a**). The latter results suggested that parkin's structure played a role in this activity, which was not solely due to the presence of its RING domains, each of which chelates two Zn^{2+} ions (**Extended Data Fig. 3.1g**)²³⁴. Nevertheless, competitive chelation of Zn^{2+} ions by rising ethylenediaminetetraacetic acid (EDTA) concentrations diminished r-parkin's antioxidant activity. This was partially reversed when excess Zn^{2+} was added back to the reaction mix (**Extended Data Fig. 3.2g**). Of note, EDTA and NEM alone had no effect on the assay (**Extended Data Fig. 3.2h**). We concluded that parkin's H_2O_2 -

reducing activity was not enzymatic, required integrity of its tertiary structure, depended on free thiols, and resembled GSH's mechanism of action.

***PRKN* expression lowers hydrogen peroxide in the cytosol**

We next asked whether parkin contributed to redox balance in cells and *in vivo*. Under basal conditions, HEK293-parkin cell lysates showed a trend toward lower levels of H₂O₂ when compared to controls; the difference became significant when we examined intact cells (**Fig. 3.2b**; **Extended Data Fig. 3.2i**). When HEK293 cultures were exposed to rising oxidative stress, *e.g.*, after CCCP treatment or excessive levels of H₂O₂ in the presence of aminotriazole (AT, a catalase inhibitor; **Extended Data Fig. 3.2a**), parkin-expressing cell lysates showed a significantly lower level of ROS (P<0.0001; **Fig. 3.2b**).

We next tested parkin's effects on H₂O₂ concentrations in mouse brain using a validated AmplexRed assay (**Extended Data Fig. 3.2j**). We found that basal ROS levels did not differ between adult *prkn*^{-/-} and WT mice, neither in the cytosol nor in isolated mitochondria (**Fig. 3.2c,d**; **Extended Data Fig. 3.3b**). Exposure to MPTP caused a significant elevation in endogenous ROS levels in parkin-deficient mice (P<0.001; **Fig. 3.2e**). Under basal conditions H₂O₂ levels were also higher in the cytosol of *Sod2*^{+/-} mutant and bi-genic animals (*prkn*^{-/-}/*Sod2*^{+/-}), which we had created to further augment stress (P<0.05; **Fig. 3.2c**; **Extended Data Fig. 3.1h,i**). Of note, at this age *Sod2*^{+/-} and bi-genic mice did not show detectable cell loss (not shown). Unexpectedly, ROS levels remained the same in isolated brain mitochondria from *Sod2*^{+/-} mice (**Fig. 3.2d**), which suggested rapid shuttling of superoxide into the cytosol³⁷⁸.

***Prkn* gene expression lowers oxidative stress in mammalian brain**

We next examined the degree of protein carbonylation, a marker of chronically elevated H₂O₂, in the same mouse lines. Carbonyl content was increased in the cytosol of *prkn*-mutant brain, even under basal conditions, consistent with a previous report by Palacino et al., who had used a different mouse model⁹⁴, but they remained normal in isolated mitochondria. We also observed a *prkn*-null allele dosage effect (**Fig. 3.2f**). As expected, carbonyl content was further increased in the bi-genic mice (P<0.05; **Fig. 3.2g**). A second marker of oxidative stress-induced protein modification, *i.e.*, nitrotyrosination, was also increased in the cytosol of *prkn*^{-/-} mice including in the heart (**Extended Data Fig. 3.3c,d**).

We next examined protein carbonyl content in patients with *PRKN*-linked ARPD vs. control subjects (each, n=4). These had been matched for age, *post mortem* interval and ethnicity (**Extended Data Fig. 3.3a**)²⁵². In the absence of parkin, protein carbonyl levels showed a trend toward elevation. Cortices from three, non-*PRKN*-linked parkinsonism cases revealed the same degree of carbonyl content as age-matched controls (**Fig. 3.2h**). Using a linear regression model with the protein carbonyl content in all brains (mouse and human) as the dependent variable, we found that oxidative stress correlated with parkin deficiency; the co-efficient was -0.3754 (95% CI, -0.6611–0.0053; P=0.05; **Fig. 3.2i**). Together, these results established that WT parkin contributed to H₂O₂ reduction in cells and brain during basal activities and with rising oxidative stress.

***Prkn* gene expression affects redox-dependent enzymatic activity**

To link these findings back to proteomic changes downstream of parkin deficiency, we explored redox-dependent enzymatic activities. In the brains of *prkn*^{-/-} mice, we found significantly increased activity of aconitase-2 (Aco2), a NADP⁺-linked enzyme, under basal conditions when compared to littermates (P<0.001; **Fig. 3.2j**). Following exposure of isolated brain mitochondria to H₂O₂, Aco2 and creatine kinase (mtCK) activities were decreased, as expected; however, their activities remained measurably higher in *prkn*^{-/-} mitochondria (P<0.05 and P<0.01, respectively; **Fig. 3.2j**; **Extended Data Fig. 3.3e**). Of note, levels of detectable Aco2 and mtCK proteins were not altered in the mitochondria of murine and human brains, when comparing WT vs. mutant genotypes (**Extended Data Fig. 3.3b,f-h**). We suspected that cytosolic redox effects in *prkn*^{-/-} mice, such as changes in the thiol network, contributed to the differences in mitochondrial enzyme activities.

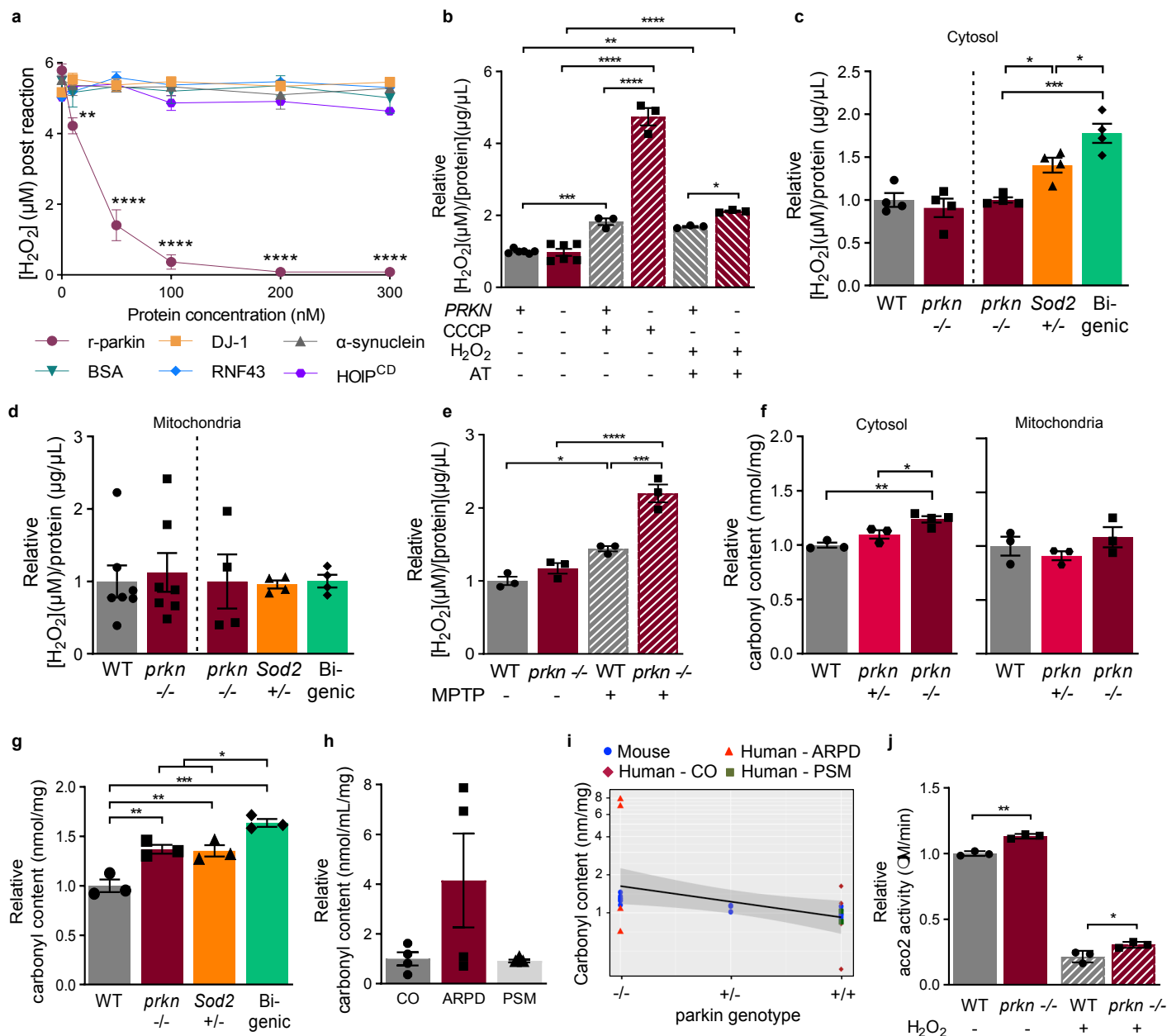
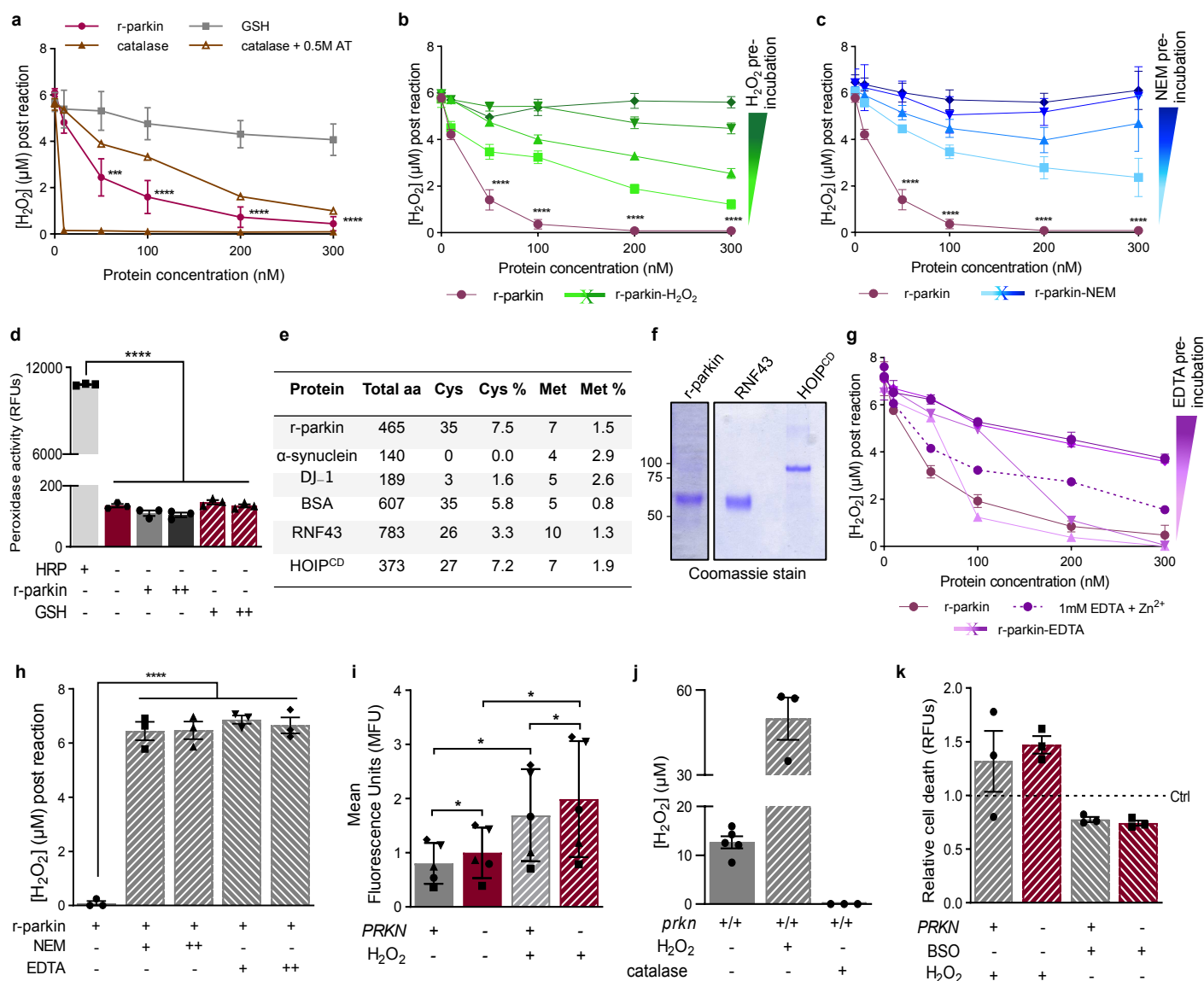


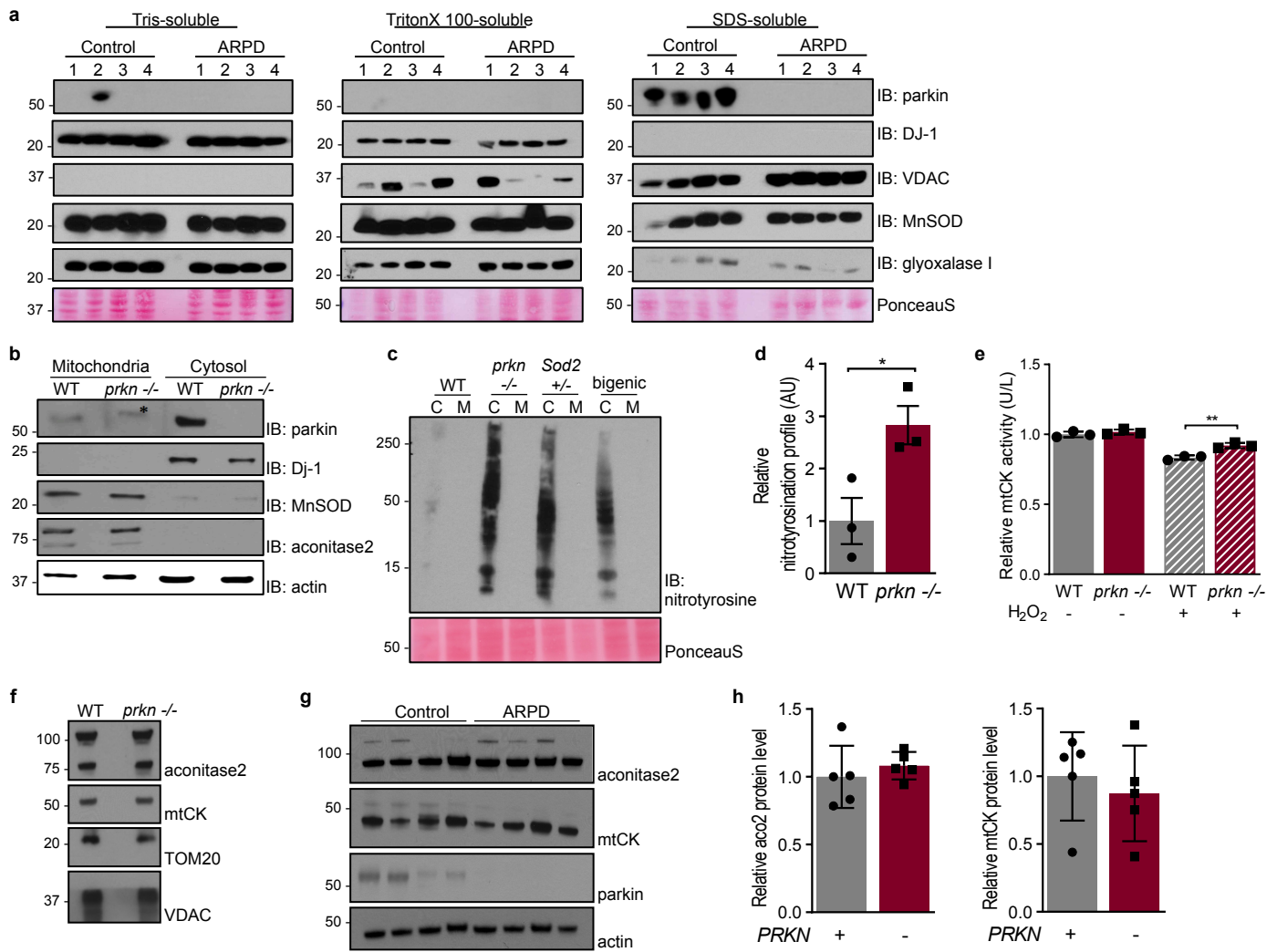
Figure 3.2: Parkin cysteines reduce hydrogen peroxide to lower oxidative stress.

a, Parkin's reducing capacity compared to DJ-1, α -synuclein, bovine serum albumin (BSA), and two RING-carrying ubiquitin ligases (RNF43 and HOIP^{cd}, (cd - catalytic domain)). **b**, Endogenous levels of H_2O_2 in HEK293-parkin and control cells under control vs. oxidizing conditions [10 μM CCCP for 1hr; 0.5M AT (to inhibit catalase) + 2mM H_2O_2 for 30mins]. Endogenous levels of H_2O_2 in **c**, cytosol-enriched and in **d**, mitochondria-rich brain homogenates of 6-8 mths-old WT and *prkn*^{-/-} and, 2-4 mths-old *prkn*^{-/-}, *Sod2*^{+/-} and bi-genic (*prkn*^{-/-}/*Sod2*^{+/-}) C57Bl/6 mice, and in **e**, 6-8 mths-old WT or *prkn*^{-/-} mouse brains treated *in vivo* with either saline or MPTP toxin. Protein carbonyl content in **f**, cytosol-enriched and mitochondria-rich brain homogenates from 8 mths-old WT, *prkn*^{+/-}, and *prkn*^{-/-} mouse brains, **g**, brain lysates of 6 mths old WT, *prkn*^{-/-}, *Sod2*^{+/-} and bi-genic mice, and **h**, aged-matched cortex specimens from controls, *PRKN*-deficient ARPD patients and subjects with other forms of parkinsonism (PSM). **i**, Carbonyl content (nmol/mL/mg) in mammalian brains of different *PRKN* genotypes. Linear regression line (blue) with standard error (gray shade) signals the overall trend. **j**, Aconitase-2 enzyme activity in mitochondria isolated from 12 mths-old WT and *prkn*^{-/-} mouse brains. 1-way or 2-way ANOVA was used for statistical analysis (* = < 0.05; ** = < 0.01; *** = < 0.001; and **** = < 0.0001).



Extended Data Figure 3.2: Parkin cysteines reduce hydrogen peroxide to lower oxidative stress.

Parkin's reducing capacity **a**, compared to glutathione and catalase, at equal molar concentrations (AT was used to block catalase activity), **b**, post oxidation with increasing concentrations of H_2O_2 ($2\mu M$ to $2mM$), and **c**, after incubation with increasing concentrations of NEM ($100\mu M$ to $1mM$). **d**, Parkin's and glutathione's peroxidase activity in comparison to horseradish peroxidase. **e**, Table summarizing the cysteine and methionine content of parkin, DJ-1, α -synuclein, BSA, RNF43 and HOIP^{cd}. **f**, r-parkin, RNF43 and HOIP^{cd} proteins visualized by Coomassie stain. **g**, Parkin's reducing capacity post incubation with increasing concentrations of ethylenediaminetetraacetic acid (EDTA) ($100\mu M$ to $1mM$). **h**, H_2O_2 reducing capacity for NEM and EDTA. **i**, H_2O_2 levels in live HEK293-parkin or control cells treated with 0 or $2mM$ H_2O_2 (flow cytometry). Paired data are represented by a distinct symbol. **j**, AmplexRed assay sensitivity demonstrated by the addition of H_2O_2 or catalase to mouse brain homogenates. **k**, Cell cytotoxicity in CHO-parkin or control cells with the addition of $2mM$ H_2O_2 or $20mM$ BSO alone. A 1way, or 2way, unpaired or paired, ANOVA with Bonferroni post hoc analysis were used for statistical analysis (*= <0.05; **= < 0.01; ***= <0.001; and ****= <0.0001).



Extended Data Figure 3.3: *PRKN* expression alters glutathione metabolism in cells and human brain.

a, Representative Western blots of parkin, DJ-1, VDAC, MnSOD, and glyoxalase-1 in Tris-soluble, TritonX 100-soluble and SDS-soluble fractions of aged-matched control and human ARPD (*PRKN* mutant) cortices. **b**, Representative Western blots of parkin, DJ-1, MnSOD, and aconitase-2 in mitochondria-enriched vs. cytosol fractions from WT and *prkn*^{-/-} mouse brains. (*) Non-specific band. **c**, Representative Western blots of nitrotyrosination in brain homogenates from 6 mths-old WT, *prkn*^{-/-}, *Sod2*^{+/-} and bi-genic mice. **d**, Quantification of nitrotyrosination signals in WT and *prkn*^{-/-} mouse hearts. **e**, Creatine kinase (mtCK) enzymatic activity in mitochondria isolated from 12 mths-old WT and *prkn*^{-/-} mouse brains. Representative Western blots of aconitase-2, mtCK, TOM20, VDAC and parkin in mitochondrial extracts from **f**, mouse brains, and **g**, human brains. **h**, Quantification of aconitase-2 and mtCK signal distribution in mammalian brains (mouse and human). A Student t-test or 1way ANOVA was used for statistical analysis (* = < 0.05; and ** = < 0.01).

Parkin contributes to the thiol network during oxidative stress

To test whether parkin contributed to the thiol network, we first compared survival rates of CHO-parkin cells following exposure to H₂O₂ after depletion of GSH with buthionine sulfoximine (BSO). Treatment with either H₂O₂ or BSO alone did not cause cell death (**Extended Data Fig. 3.2k**). Under combined stress conditions, *PRKN* expression was protective: it led to significantly less cell death ($P < 0.05$; **Fig. 3.3a**) and normalized ROS levels ($P < 0.001$; **Fig. 3.3b**), which resulted in parkin oxidation (Fig. 1a). Treatment with excess n-acetylcysteine, an abundant source of thiols, resulted in similarly protective effects as *PRKN* expression (**Fig. 3.3a,b**). We conjectured that parkin supplements the cellular thiol network that neutralizes ROS through its own cysteines.

***PRKN* gene expression alters glutathione redox state in cells and human brain**

We examined this by directly measuring levels of GSH (reduced) and GSSG (oxidized) by HPLC. There, CHO-control cells showed high concentrations of GSH and low amounts of GSSG with an elevated GSH:GSSG ratio (**Fig. 3.3c**). Increased expression of WT parkin significantly reversed these redox indices ($P < 0.01$), but no change was found in the total concentration of GSH and GSSG (**Extended Data Fig. 3.4a**). Consistent with reports of CHO cells' high stress tolerance³⁷⁹, exogenously added H₂O₂ did not change the redox indices between CHO-control and CHO-parkin cells with the exception of now equalized GSSG levels (**Fig. 3.3d**). Parkin-dependent changes in GSH levels and the GSH:GSSG ratio were also observed in HEK293 cells (not shown) and human, dopamine SH-SY5Y cells (**Fig. 3.3e**).

The interplay between *PRKN* expression and GSH redox state was validated in human brain using cortex specimens from patients with *PRKN*-linked ARPD and healthy controls. In the absence of detectable parkin, reduced GSH levels as well as the ratio of GSH:GSSG were significantly increased ($P < 0.05$). No differences were seen for GSSG levels (**Fig. 3.3f**) or the total concentration of GSH and GSSG (**Extended Data Fig. 3.4b**). The redox changes recorded in human brain closely mirrored those in CHO cells under oxidative stress (**Fig. 3.3d**). We concluded that a reciprocal relationship existed between *PRKN* expression and GSH levels *in vivo*. When using a linear regression model with GSH levels as the dependent variable, we found that lower expression of parkin was associated with higher GSH levels; the co-efficient was -0.6889 (95% CI, -0.849 to -0.411; $P < 0.0001$; **Fig. 3.3g**).

***Prkn* deletion alters glutathione metabolism in mice with a mitochondrial defect**

Two groups previously reported elevated GSH levels in *prkn*^{-/-} mice and their glia^{76,300}. When examining the same colony as Itier et al. using our HPLC method, parkin-deficient brains showed non-significant trends for thiol changes, *i.e.*, higher concentrations of GSH, lower levels of GSSG, and a higher GSH:GSSG ratio (**Fig. 3.3h**). Brains from *Sod2*^{+/-} haploinsufficient animals showed identical results. However, in bi-genic (*prkn*^{-/-} // *Sod2*^{+/-}) animals, the same changes in redox indices were detected ($P < 0.05$; **Fig. 3.3h**), as measured in human parkin-deficient cells and cortices. These results established a relation between parkin protein expression, glutathione redox state and degree of oxidative stress *in vivo*. We next studied potential mechanism(s) to explain it.

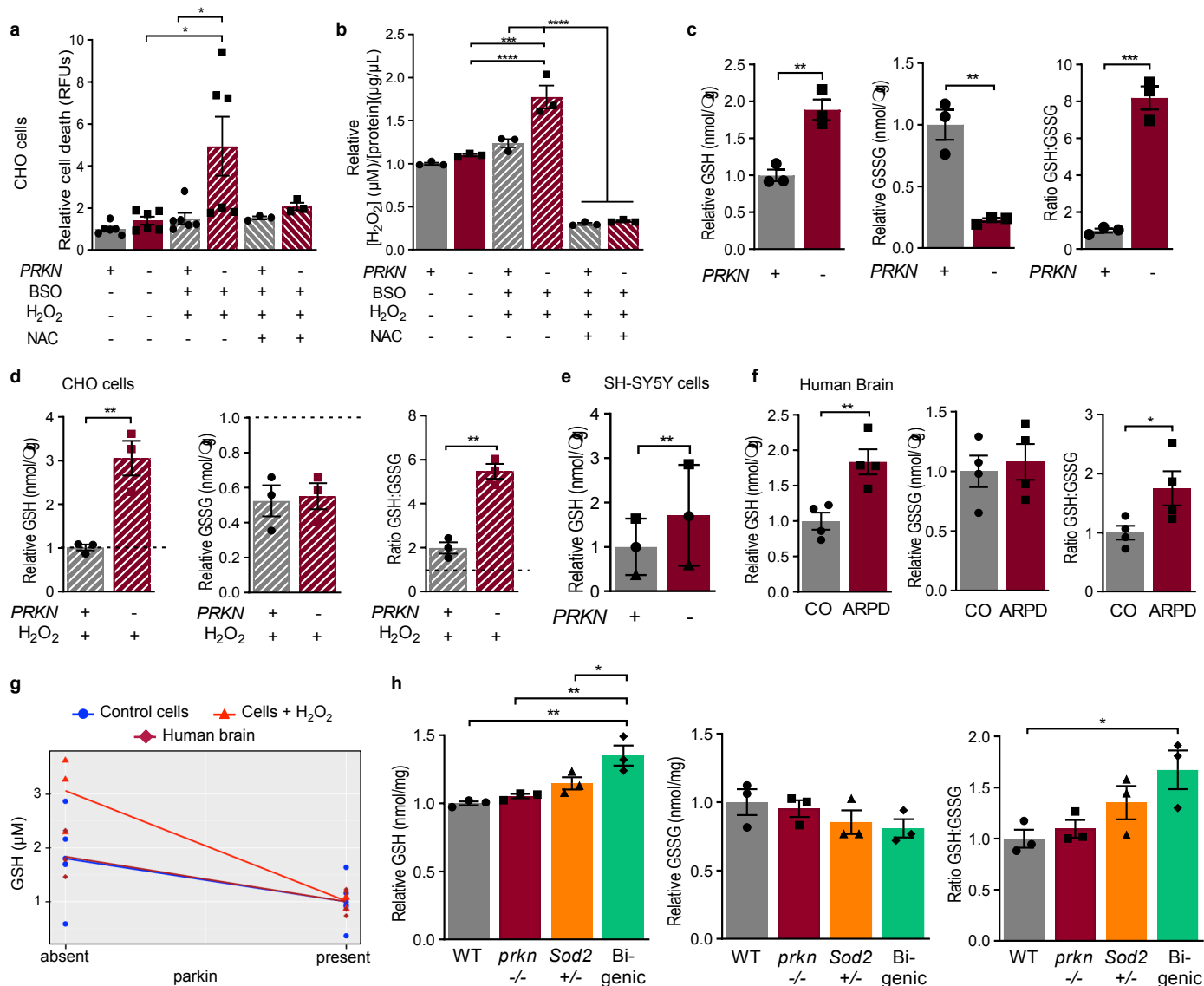


Figure 3.3: *PRKN* expression alters glutathione metabolism in cells and human brain.

Cellular toxicity **a**, and endogenous H₂O₂ levels **b**, in CHO-parkin and control cells under control or oxidizing conditions (2 mM H₂O₂ + 2mM buthionine sulfoximine (BSO)), with or without supplementation by 20mM n-acetylcysteine (NAC). Reduced GSH, oxidized GSSG, and GSH:GSSG ratio in CHO-parkin or control cells under **c**, control conditions, and **d**, post H₂O₂ stress (HPLC method). **e**, GSH levels in SHSY5Y cells under control conditions (monochlorobimane (MCB) method). Paired data are represented by a distinct symbol. GSH, GSSG, GSH:GSSG ratio in **f**, control and *PRKN* deficient human brain cortices, and **h**, brains of 6 mths-old WT, *prkn* -/-, *Sod2* +/- and bi-genic mice (HPLC method). **g**, GSH (μM) levels in several paradigms of *PRKN* genotype comparisons, as indicated. Linear regression lines represented the overall trend of control cells (blue), cells + H₂O₂ (red), and human brain (darker red). A Student t-test or 1-way unpaired or paired ANOVA were used for statistical analysis (* = < 0.05; ** = < 0.01; *** = < 0.001; and **** = < 0.0001).

***De novo* synthesis of glutathione is not altered in parkin-deficient brain**

We first examined GSH *de novo* synthesis. When measuring the total pool of glutathione (GSH and GSSG) by HPLC in our parkin-deficient vs. WT parkin expression paradigms, we saw no difference in the total concentration across cell cultures, murine brains and human cortices (**Extended Data Fig. 3.4a,b,c**). In accordance, we found no difference in transcript numbers for the rate-limiting enzymes in GSH synthesis, *i.e.*, the glutamate-cysteine-ligase-catalytic (*GCLC*) and glutamate-cysteine-ligase-modifier (*GCLM*) subunits³⁸⁰, or for *Dj-1*, in these lines (**Extended Data Fig. 3.4d,e**). Unexpectedly, in bi-genic mice, where we recorded a significant rise in the total glutathione (GSH+GSSG) concentration ($P<0.05$), we also found no change in numbers for *GCLC* or *GCLM* mRNA (**Extended Data Fig. 3.4c,e**). Thus, *de novo* synthesis from transcription did not explain the glutathione redox changes seen in parkin's absence.

Glutathione recycling is altered in *prkn*-null brain

We next explored a second mechanism, *i.e.*, enhanced GSH regeneration by glutathione reductase (GR). This enzyme is activated by rising levels of ROS or its substrate (GSSG), and is inhibited by excess product levels, such as GSH or NADP^+ ³⁸¹⁻³⁸³. By using the Tietze method, which utilizes exogenously added GR activity to indirectly measure GSH and GSSG levels, we reproduced the same significant changes, *i.e.*, GSH elevation ($P<0.01$), GSSG lowering ($P<0.01$) and a rise of the GSH:GSSG ratio, in *prkn*^{-/-} mice as found in cells and human brain (**Fig. 3.4a** and **Fig. 3.3c-f**, respectively). These findings in mouse brain matched the results by Itier et al.; they also suggested that GR activity was indeed higher in parkin's absence. We reasoned that activation of endogenous GR was

even more elevated in ARPD cortices than in *prkn*^{-/-} brain, because GSH levels were higher in the former than the latter using the GR-independent HPLC method (**Fig. 3.3f-h**).

Parkin contributes to the pool of recycled glutathione

We next explored whether parkin had any direct effects on thiol network changes, such as via GSH recycling. We first tested this using recombinant proteins, both tagged and untagged, and eosin-labelled GSSG (*i.e.*, E-GSSG-E, referred to as Di-E-GSSG) (**Extended Data Figs. 3.2f,4f**). We discovered that full-length, human parkin had concentration-dependent activity in reducing Di-E-GSSG to the fluorescence-emitting E-GSH (545 nm) (**Fig. 3.4b**). Of note, Di-E-GSSG itself is non-fluorescent³⁸⁴. N-terminally truncated parkin comprising the IBR-RING2 domains (aa 327-465) and a C-terminal RING2 peptide (aa 425-465) also showed E-GSH-regenerating activity (**Extended Data Fig. 3.4f,g**; and not shown). In contrast, the control protein-tag alone (*e.g.*, maltose-binding protein (MBP); ~50 kDa) and incubation with E-GSH (without Di-E-GSSG present) showed no activity (**Fig. 3.4b**).

Resulting from this biochemical reaction, the formation of E-S-glutathionylated parkin (referred to as parkin-SG-E) was confirmed by using MBP-parkin proteins, non-reducing SDS/PAGE analyses and UV light. We found that SG-E-carrying parkin proteins were only visible under UV light after the incubation of nascent preparations with Di-E-GSSG (*e.g.*, **Extended Data Fig. 3.4h**, lane 2 of left panel). Furthermore, the incubation of MBP-IBR-RING2-SG-E and MBP-parkin-SG-E with either DTT by itself or with

activated glutaredoxin-1 or -2 (Grx1, Grx2)³⁸⁴ reversed the S-glutathionylation of parkin; the latter confirmed that oxidation in the form of disulphide-bond formation had occurred during the earlier reaction (**Extended Data Fig. 3.4h**). Parkin proteins without an -SG-E modification did not interact with Grx1, Grx2, or thioredoxin-1 (Trx1; not shown). We reasoned that parkin's ability to interact with GSSG could alter the GSH:GSSG ratio.

To test this, we incubated untagged, human r-parkin (1mM) with eosin-free ratios of [GSH]:[GSSG] at a total concentration of 10mM per well (**Fig. 3.4c**). Following its incubation with GSSG, we determined that under these conditions parkin was able to reduce up to 5 equivalents to GSH (6.8μM GSH minus a baseline signal of 1.3μM for r-parkin alone; **Fig. 3.4c**). Other ratios of [GSH]:[GSSG] showed corresponding changes. Therefore, for every recycled GSSG molecule one reduced GSH and one S-glutathionylated cysteine on parkin had been generated. These *in vitro* findings demonstrated a direct biochemical effect by parkin onto its surrounding thiol network.

We next sought to map specific S-glutathionylation sites on parkin using MBP-parkin-SG-E preparations by mass spectrometry, which was performed under non-reducing conditions. MBP-parkin-SG-E was subjected to trypsin digestion followed by LC-MS/MS and MALDI analyses. We detected two residues that were consistently S-glutathionylated, *i.e.*, the conserved cysteine 59 and human-specific cysteine 95, each carrying an extra mass of 305.0682 (**Fig. 3.4d,e**), which corresponded to S-linked glutathionylated oxidation. A third, less frequently identified residue to be S-glutathionylated was cysteine 377 within parkin's IBR domain. We concluded from these

results that S-glutathionylation represents a *bona fide* oxidative modification of human parkin, which arises from its interaction with GSSG.

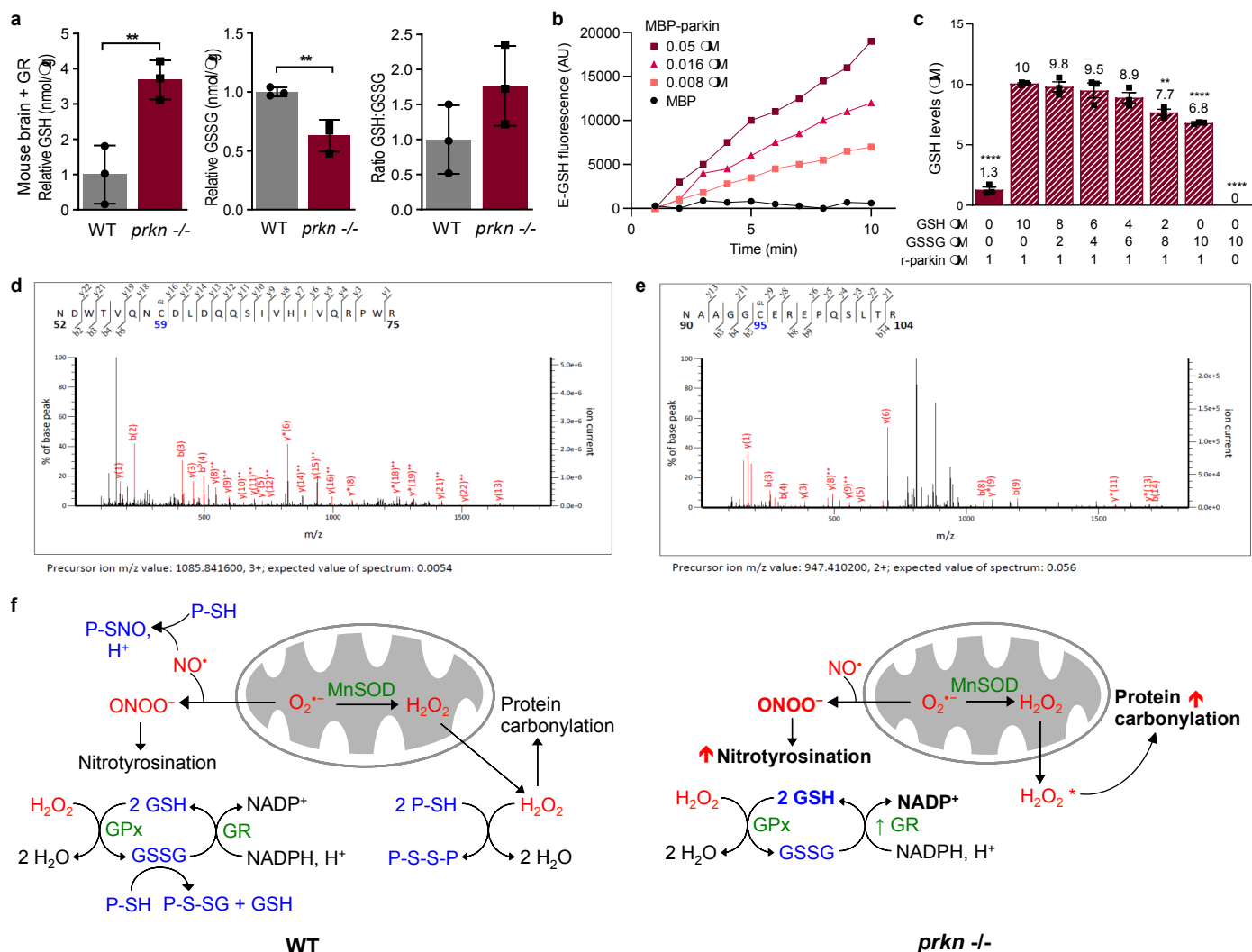
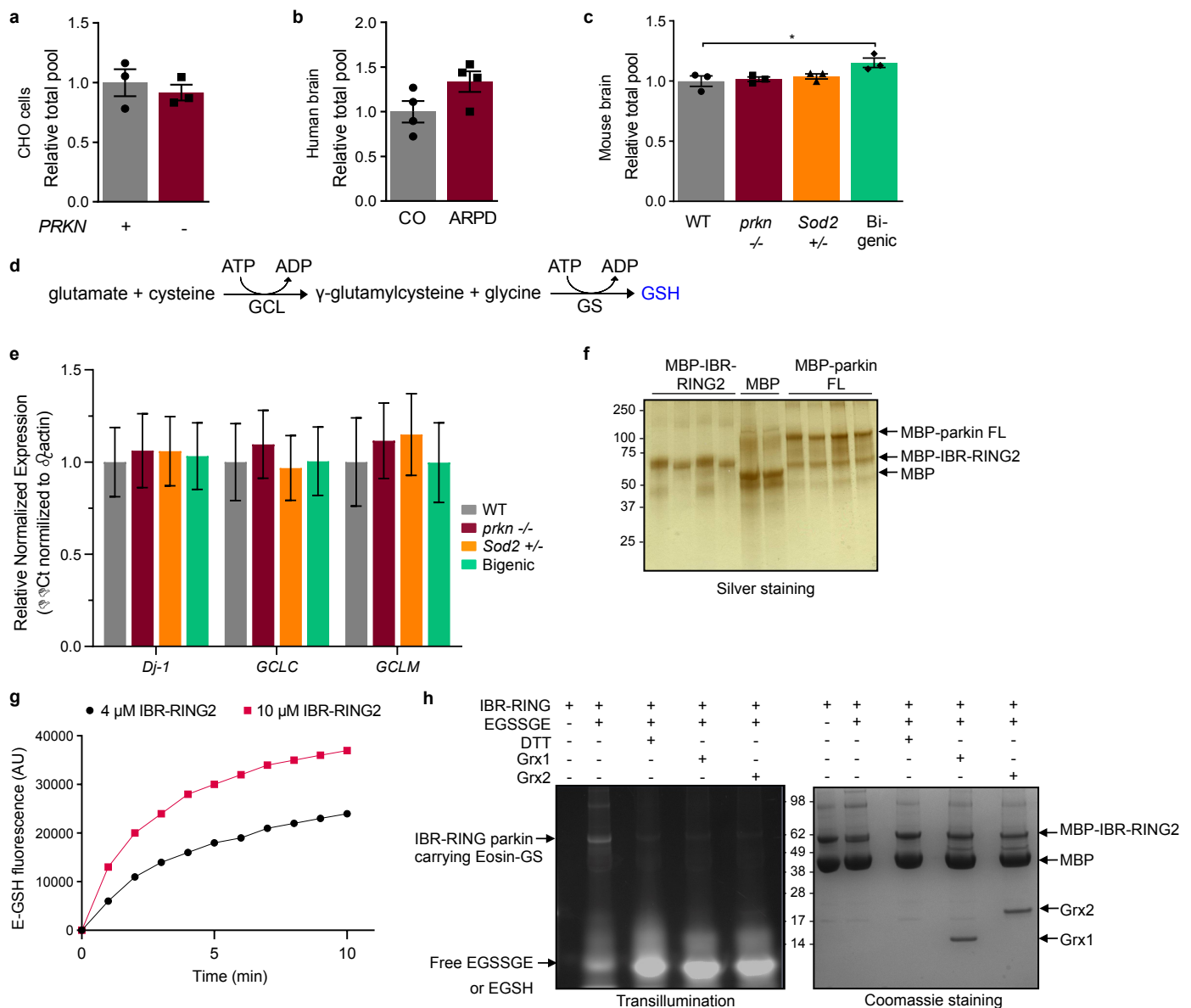


Figure 3.4: Parkin contributes to glutathione recycling independent of *de novo* synthesis.

a, GSH, GSSG, and GSH:GSSG ratio in 11-13 mths-old WT and *prkn*^{-/-} mouse brains (GR-dependent Tietze method). **b**, E-GSH release after exposure of increasing concentrations of full-length MBP-parkin to 20μM Di-E-GSSG. **c**, GSH levels (μM) of various GSH:GSSG ratios incubated with 1μM of full-length, untagged r-parkin. A Student t-test or 1way ANOVA was used for statistical analysis (** = < 0.01; and *** = < 0.001). **d**, Human parkin peptide aa52-75 is shown, with cysteine 59 S-gluthionylated. **e**, Human parkin peptide aa90-104 is shown, with cysteine 95 S-gluthionylated. **f**, Graphical depiction of redox changes including: GSH recycling; generation of ROS levels (*i.e.*, H₂O₂; superoxide) leading to carbonylation; metabolism of nitric oxide (NO) leading to nitrotyrosination; and function of select enzymes in WT *vs.* parkin-deficient (*prkn*^{-/-}) mammalian brain. P, parkin; MnSOD, Mn²⁺-dependent superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase. * Indicates evidence from human brain.



Extended Data Figure 3.4: Parkin contributes to glutathione recycling independent of *de novo* synthesis.

The total GSH + GSSG pool in **a**, CHO cells, **b**, human cortices, and **c**, brain homogenates of 6 mths-old WT, *prkn*^{-/-}, *Sod2*^{+/-}, and bi-genic mice (HPLC method). A 1 way ANOVA was used for statistical analysis (*= <0.05). **d**, Graphic depiction of GSH synthesis from glutamate and cysteine, with glutamate cysteine ligase (GCL) as the rate-limiting enzyme. **e**, *Dj-1*, glutamate cysteine ligase catalytic subunit (*GCLC*) and glutamate cysteine ligase modifier subunit (*GCLM*) gene expression in brains of 6 mths-old WT, *prkn*^{-/-}, *Sod2*^{+/-} and bi-genic mice. **f**, Silver stain of MBP-tag (~45-50 kDa), MBP-IBR-RING2 (parkin, aa 327-465, ~60-65 kDa), and MPB-parkin (~90-95 kDa). **g**, E-GSH release post treatment of increasing concentrations of MBP-IBR-RING2 protein with 20 μ M Di-E-GSSG. **h**, Representative UV transillumination and Coomassie Blue stain gels of S-glutathionylated MBP-IBR-RING2 protein. Results are representative of three independent experiments. Grx1, glutaredoxin-1; Grx2, glutaredoxin-2; EGSSGE, Di-E-GSSG (eosin-labelled oxidized glutathione).

Discussion

Our study generated four novel insights into the protective effects of PD-linked parkin:

One, we restaged in cell models and mouse brain the age-associated loss of parkin solubility that occurs in human brain^{332,373} by inducing its progressive oxidation using mitochondrial and cytosolic stressors (**Fig.1**). We then monitored for specific cysteine oxidation sites in murine parkin using LC-MS/MS (**Extended Data Table 3.1**). There, we found a relative loss of free thiols in parkin associated with the rise of oxidative stress in MPTP-treated mice. We also mapped several cysteines that had undergone irreversible oxidation, such as to sulfinic and sulfonic acid, under both basal and oxidizing conditions, *e.g.*, C211, C195, C237, C252, and C376. We noticed with interest –given the role of parkin’s phosphorylation by ARPD-linked PINK1 in *ex vivo* experiments during mitophagy³⁶⁵, that S77 of murine parkin was found phosphorylated in MPTP-exposed brain (**Extended Data Table 3.1**).

Two, we established that *PRKN* expression contributes to cytosolic ROS neutralization and *in vivo* redox homeostasis; this, to limit oxidative stress in high energy-demanding organs, such as the brain and heart. These anti-oxidant effects are mediated by parkin’s cysteines, which are sensitive to oxidizing as well as reducing environments (**Fig. 3.2**). We found that parkin contributes to redox homeostasis during ongoing oxidative stress production, as monitored by the carbonyl content in mammalian brain (**Fig. 3.2**). We also provided evidence that parkin’s modulation of cytosolic ROS levels affects the activities of redox-sensitive enzymes within mitochondria (*e.g.*, Aco2 and mtCK; **Fig. 3.2**). This finding thus helps explain the observation made by Berger et al., who had postulated an

‘intrinsic, pro-mitochondrial effect by cytosolic parkin’²⁸⁹. We suspect that the structural changes in redox-sensitive proteins from *prkn*^{-/-} mice^{94,95} were mediated by the net reduction in free thiols, thereby not only increasing protein susceptibility to endogenously generated oxidative radicals (**Figs. 3.2, 3.3**), but also altering overall redox balance (including co-factors) within the cell.

Three, parkin’s cellular protection from ROS appeared independent of E3 ligase activity. Its oxidation had previously been widely interpreted as a loss of its function^{256,262}. Parkin’s redox-regulating activity may not be mutually exclusive from its E3 ligase function. It is known that low levels of oxidation can activate E3 ligase activity *ex vivo*. Of note, both cellular redox-regulation and E3 ligase function are thiol-based^{256,258,260,357}. Furthermore, the reversible nature of some of the oxidative modifications identified here suggests that WT parkin could continuously undergo redox alterations *in vivo*. We propose that distinct cellular conditions may require more of parkin’s redox functions, such as during elevated oxidative stress, whereas others may demand more E3 activity, such as during organelle-specific autophagy. Because powerful oxidative stressors are required to recapitulate in mice parkin’s aggregation and insolubility (**Fig. 3.1**), which occurs progressively during human brain ageing³⁷³, we speculate that parkin is exposed to even higher pro-oxidant conditions in long-lived primates.

Four, we uncovered a reciprocal relationship between *PRKN* expression and glutathione metabolism *in vivo*. The thiol network, to which we propose parkin contributes, is critical in the maintenance of overall redox balance, and thus, to cellular and organ health^{339,340}. In parkin’s absence: GSH is elevated; GSSG levels are diminished; the ratio of

GSH:GSSG is increased; and total glutathione levels (GSH+GSSG) are unchanged, except in bi-genic mice (**Fig. 3.3**). We postulate that parkin's presence reduced cellular needs for higher levels of free GSH. We first explored *de novo* synthesis of GSH but found no increase in *GCLM* or *GCLC* mRNA levels. As for the rise in total glutathione in bi-genic mice, we reasoned that higher GSH levels may still have resulted from *de novo* synthesis but via more effective translation of existing *GCLM* mRNA^{380,383}. Using the Tietze-method to analyze murine brain, we observed an increase in GSH and reciprocal decrease in GSSG levels in *prkn*^{-/-} mice, which was therefore attributable to increased GR enzyme-related activity (**Fig. 3.4**)^{382,383}. Informed by the effect of GR activity, we next explored whether parkin itself contributed to increased GSH levels by direct thiol-mediated recycling of GSSG. There, we found that parkin bound GSSG in a concentration-dependent manner, which resulted in one GSH and one of parkin's cysteines S-glutathionylated per GSSG recycled (**Fig. 3.4**).

The finding of parkin S-glutathionylation (**Fig. 3.4**), and its reversal by glutaredoxin-1 and -2, further highlights the relevance of parkin's integration into redox chemistry. We noted with interest that primate-specific cysteine 95 is targeted for oxidation by both S-glutathionylation (**Fig. 3.4**) and dopamine radical adduct formation³⁷³. We question whether this specific residue, which is located in the linker region of human parkin, could have an effect on parkin activation akin to S65 phosphorylation during mitophagy³⁶⁵. We anticipate that the many posttranslational modifications of parkin, which we have identified here, will inform structural studies and cell biology research in the future. We propose that these should be pursued in the context of parkin's redox chemistry.

Future studies will also explore which region and which cell type in the nervous system⁷⁶ contribute most significantly to the parkin-dependent glutathione redox changes we have observed. The lack of significantly elevated GSH levels in *prkn*^{-/-} mouse brain by HPLC suggests that this species can compensate for increased cytosolic ROS via other redox state-regulating mechanisms, such as via glutathione peroxidase (GPx) activity (**Fig. 3.4f**). High tolerance to ROS may help explain the lack of dopamine cell loss in *prkn*-null mice^{217,299,300}. In aged mouse brain, one or more additional stressors could be required, such as demonstrated in the *prkn*^{-/-}/*Sod2*^{+/-} mice. A human-specific redox aspect is that nigral neurons experience high levels of ROS production, dysregulated iron homeostasis, and more radical formation from oxidative dopamine metabolism^{373,385,386}.

The strengths of our investigations are three-fold: one, results in cellular and murine models restaged the findings in human tissues^{332,373} (**Figs. 3.2h,i; 3.3f,g**); two, our research integrated *in vitro*, *ex vivo*, *in situ* and *in vivo* paradigms; and three, the redox hypothesis of parkin function readily explains its many reported roles in distinct cellular paradigms, *e.g.*, cancer biology, mitochondrial health, immunity, etc.³⁸⁷. These pathways are often regulated by redox chemistry (**Fig. 3.4f**).

We have also identified shortcomings. In future work we will conduct a more extensive assessment of the diversity and quantity of oxidative modifications in brain parkin by LC-MS/MS, for example in our MPTP-exposed *vs.* untreated mouse model, in order to map additional *bona fide* oxidation sites that occur *in vivo*. Due to the transient nature of S-glutathionylation (as well as of S-nitrosylation²⁵⁸) and LC-MS/MS methods employed

here (mostly reducing conditions), we have not yet validated parkin's S-glutathionylation *in vivo*. Furthermore, we wish to study a greater number of *PRKN*-genotyped, age-matched, human brain tissue specimens to quantify protein carbonylation and nitrotyrosination, as well as to explore human brain-specific changes in redox state-related transcriptomes. We have plans to characterize additional glutathione-regulating enzyme levels *in vivo*; this, to validate our model that GR activity is markedly increased in ARPD brain (**Fig. 3.4f**).

Our discovery that parkin's seemingly diverse activities are anchored in traditional redox chemistry provides a biochemical and unifying explanation for its role in neuroprotection. It may help answer the two decades-old question: "What function of parkin is essential in conferring the selective protection of dopamine neurons in the human brainstem?"^{8,373} Further exploration of parkin's redox biology should create new opportunities to develop urgently needed therapies for patients with young-onset parkinsonism.

Materials And Methods

Mouse tissues

Brains and hearts were collected from wild-type C57Bl/6J from Jackson laboratories, *prkn*-null from Dr. Brice's laboratory³⁰⁰, *Sod2* +/- mice from Jackson laboratories; the bi-genic mouse (*prkn*^{-/-}//*Sod2*^{+/-}) was created by crossing *prkn*-null mice with *Sod2* haploinsufficient mice, and interbreeding heterozygous offspring. The bi-genic mouse will be further characterized elsewhere (El Kodsi et al., in preparation). Mouse brains collected were homogenized on ice in a Dounce glass homogenizer by 20 passes in Tris salt buffer with or without the addition of 1% H₂O₂, transferred to ultracentrifuge tubes and spun at 55,000 and 4°C for 30 mins to extract the soluble fraction. The resulting pellets were further homogenized in the tris salt buffer with the addition of 2-10% SDS, transferred to ultracentrifuge tubes and spun at 55,000 rpm and 10°C for 30 minutes to extract the insoluble fraction.

Cell culture, transfection and oxidation

Human embryonic kidney (HEK293) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 % penicillin/streptomycin and 10 % heat-inactivated fetal bovine serum (FBS) at 37°C with 5 % CO₂. Four to 15 µg of cDNA coding for N-terminally flag-tagged parkin or empty Flag control vector (pcDNA3.1) using a 1:1 ratio of cDNA:Lipofectamine 2000, was used for ectopic overexpression. The cDNA and Lipofectamine 2000 were incubated for 20 min at room temperature before being applied directly to the cells for 1 hour at 37°C with 5 % CO₂ followed by direct addition of fresh growth medium. Cells were incubated another 24 hours before treatment, harvesting and

analysis. Control Chinese hamster ovary cells, stably expressing the myc-vector (CHO), or expressing myc-parkin (CHO-parkin) were also used.

All chemicals (H_2O_2 , CCCP, NEM, IAA, DTT, AT, BSO and NAC) were added directly to cells at ~75% confluence in growth or OPTI-MEM media. Cells were manually scrapped, spun at a 1000 rpm for 5 minutes, the pellets washed with PBS and then homogenized in a Tris salt buffer, transferred to ultracentrifuge tubes and spun at 55,000 rpm and 4°C for 30 minutes to extract the soluble fraction. The resulting pellets were further homogenized in the Tris salt buffer with the addition of 2-10% SDS, transferred to ultracentrifuge tubes and spun at 55,000 rpm and 10°C for 30 minutes to extract the insoluble fraction. SH-SY5Y cells were generally seeded at a density of $0.5\text{-}1 \times 10^6$ cells/mL. Once cells reached 70-80 % confluency they were transfected with cDNA coding for C-terminally Flag-tagged Parkin or empty Flag control vector (pcDNA3) by electroporation using the nucleofector method described by Hu and Li, 2015. A total of 2 million cells were resuspended in 100 μL of OPTI-MEM containing cDNA (2 μg) and 1 % polyoxamer 188. The cells were electroporated using the X Unit and pulse code “CA-137” on a Lonza 4D-Nucleofector. Following electroporation, cells were seeded at a concentration of $0.8\text{-}1 \times 10^6$ cells/mL³⁸⁸.

Western blot and densitometry

Brain homogenates and cell lysates were run on 4-12 % Bis-Tris SDS-PAGE gels using MES running buffer. Proteins were transferred to PVDF membranes using transfer buffer, and immunoblotted for parkin, DJ-1, MnSOD, aconitase-2, creatine kinase, VDAC, TOM20, nitrotyrosine, and flag-epitopes. Actin and Ponceau S staining were

used as loading controls. For densitometry quantification, the signal intensity of parkin from each sample was measured as pixel using Image J Software, and controlled for loading, against actin's band intensity. Proteins were also stained in gel using Coomassie brilliant blue R-250 dye. The gel was first fixed in 10% acetic acid, 50% methanol, followed by staining for 2-24 hours with Coomassie and finally destained in 7.5% acetic acid, 5% methanol until crisp blue bands appeared.

MPTP treatment

Eight to 12 months old WT and *prkn*-null mice were injected intraperitoneally with 40mg/kg of saline or MPTP and sacrificed an hour later³⁷⁶. The brains were harvested for ROS measurement, protein analysis by Western blot and immunoprecipitation of parkin and mass spectrometry analysis. For mass spectrometry, the brains harvested were first incubated in IAA prior to homogenization and fractionation as described above. Brain homogenates were then incubated with anti-parkin conjugated to magnetic beads (Dynabeads Coupling Kit; Invitrogen). A magnet was used to capture parkin bound to the beads, and several washes were used to remove unbound proteins. Eluted fractions (IP elute) along with controls (input, unbound, wash and recombinant parkin protein standards) were run on SDS/PAGE and blotted with anti-parkin. A sister gel was stained with Coomassie as described above and gel slices corresponding to band sizes 50-75 kDa were excised and analyzed by LC-MS/MS, as described in detail by Tokarew et al., 2020.

Recombinant, tag-less protein expression in pET-SUMO vector

Wild-type and truncated (residues 321-465) human parkin were expressed as 6His-Smt3 fusion proteins in *Escherichia coli* BL21 (DE3) Codon-Plus RIL competent cells (New England Biolabs) as previously described^{234,364,365}. DJ-1 and SNCA coding sequences were cloned from a pcDNA3.1 vector into the pET-SUMO vector using PCR and restriction enzymes. All proteins were overexpressed in *E. coli* BL21 Codon-Plus competent cells and grown at 37 °C in 2 % Luria Broth containing 30 mg/L kanamycin until OD600 reached 0.6, at which point the temperature was reduced to 16°C. Parkin protein-expressing cultures were also supplemented with 0.5 mM ZnCl₂. Once OD600 reached 0.8, protein expression was induced with isopropyl β-D-1-thiogalactopyranoside, except ulp1 protease, which was induced once OD600 had reached 1.2. The concentration of IPTG used for each construct is as follows: 25 μM for wild-type and point mutants of parkin, and 0.75 mM for truncated parkin, DJ-1, α-synuclein, SAG, and ulp1 protease. Cultures were left to express protein for 16-20 h. Cells were then harvested, centrifuged, lysed and collected on Ni-NTA agarose beads in elution columns.

Recombinant maltose-binding protein-tagged protein expression in pMAL-2T vector

Wild-type and truncated (residues 327-465) human parkin were expressed in the pMAL-2T vector (a gift from Dr. Keiji Tanaka), as previously described³⁸⁹. Parkin produced in this vector contained an N-terminal maltose-binding protein (MBP) and thrombin cleavage site (LVPRGS). All proteins were overexpressed in *E. coli* BL21 Codon-Plus competent cells (New England Biolabs) and grown at 37 °C in 2 % Luria Broth

containing 0.2 % glucose and 100 mg/L ampicillin until OD600 reached 0.3-0.37, at which point protein expression was induced with addition of 0.4 mM isopropyl β -D-1-thiogalactopyranoside. Cultures were left to express protein at 37 °C until OD600 reached 0.9-1.0. Harvested protein isolates were purified using amylose resin in buffers containing 100 μ M zinc sulfate and 10 mM maltose.

ROS (H₂O₂) measurements of recombinant proteins, tissue and cell lysates

Amplex[®] Red hydrogen peroxide/peroxidase assay kit (Invitrogen A22188) was used to monitor endogenous levels of H₂O₂ in tissues and cells, and residual levels of H₂O₂ after incubation with recombinant parkin (WT, or pre-incubated with increasing concentrations of H₂O₂, NEM, or EDTA), DJ-1, SNCA, BSA, RNF43 (BioLegend), HOIP (Boston Biochem), GSH, catalase, NEM and EDTA for 30 minutes. Pre-weighed cortex pieces from human brains (or pelleted cells) were homogenized on ice in the 1x reaction buffer provided, using a Dounce homogenizer (3 times volume to weight ratio). Homogenates were diluted in the same 1x reaction buffer (10x and 5x). A serial dilution of the H₂O₂ standard provided was prepared (20, 10, 2 and 0 μ M). 50 μ L of standards and samples were plated in a 96 well black plate with clear flat bottom. The reaction was started by the addition of 50 μ L working solution which consisted of 1x reaction buffer, Amplex[®] red and horseradish peroxidase. The plate was incubated at room temperature for 30 minutes protected from light. A microplate reader was used to measure either fluorescence with excitation at 560 nm and emission at 590 nm, or absorbance at 560 nm. The obtained H₂O₂ levels (μ M) were normalized to the tissue weight (g) or protein

concentration ($\mu\text{g}/\mu\text{L}$). The same assay was also used to measure parkin and glutathione's peroxidase activity compared to horseradish peroxidase (HRP).

ROS measurements of intact cells

HEK293 cells were transfected with flag-parkin or control vector (pcDNA) as described above. After 24 h the cells were lifted using trypsin and re-seeded in a 12-well dish at a density of 0.3×10^6 cells/mL. After 48 h the cells were treated with 0 mM or 2 mM H_2O_2 in OPTI-MEM medium at 37°C and 5 % CO_2 . After 1 h the cells were washed with OPTI-MEM and incubated with 20 μM of dichlorofluorescein diacetate (DCFH-DA, Sigma) for 30 min at 37°C and 5 % CO_2 . Cells were collected using a cell lifter and treated with ethidium-1 dead stain (Invitrogen) for 15 min at room temperature. Samples were analyzed using a BD Fortessa flow cytometer set to measure the ROS-sensitive (DCFH-DA, ex. 488 nm and em. 527 nm) and viability (ethidium-1, ex, 528 nm and em. 617 nm) stains. The results were reported as the average mean fluorescence intensity (MFI) of ROS in live cells. Each separate transfection was considered one biological replicate.

Cell cytotoxicity assay

VybrantTM cytotoxicity assay kit (Molecular Probes V-23111) was used to monitor cell death through the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6yPD) from damaged cells into the surrounding medium. 50 μl of media alone (no cells), media from control and stressed CHO-parkin and control cells and cell lysates were added to a 96-well microplate. 50 μl of reaction mixture, containing reaction buffer,

reaction mixture and resazurin, was added to all wells, and the microplate was incubated at 37°C for 30 mins. A microplate reader was used to measure either fluorescence with excitation at 560 nm and emission at 590 nm. A rise in fluorescence indicates a rise in G6PD levels i.e. a rise in cell death.

Mitochondria isolation

Fresh tissue was cut with scissors into manageable pieces, rinsed in cold PBS, then homogenized using either a Dounce homogenizer or Warning blender in the presence of twice the tissue volume of buffer A (20mM Hepes pH 7.4, 220mM mannitol, 68mM sucrose, 80mM KCl, 0,5mM EGTA, 2mM Mg(Ac)₂, 1mM DTT, 1X protease inhibitor (Roche)). The sample was centrifuged at 4070g in a tabletop centrifuge for 20 minutes at 4°C. The supernatant was collected and spun again as above. The resulting supernatant was spun at 10 000g for 20 minutes at 4°C and the pellet was then washed in the above buffer and spun again at 10 000g for 20 minutes. The resulting mitochondrial pellet was carefully resuspended in buffer B (20mM Hepes pH 7.4, 220mM mannitol, 68mM sucrose, 80mM KCl, 0,5mM EGTA, 2mM Mg(Ac)₂, 10% glycerol), aliquoted, and snap frozen.

Aconitase assay

The Aconitase Enzyme Activity Microplate Assay Kit (MitoSciences) was used to measure aconitase activity in mitochondria isolated from wild type and parkin knock-out mouse brains as per manufacturer's instructions. Two brains each from 12 months old mice were pooled to provide the mitochondria samples and normalized for total protein

concentration. These were treated with 0 or 4 mM H_2O_2 just prior to assay. The catalytic conversion of isocitrate to cis-aconitate by aconitase was measured by quantifying the amount of cis-aconitate in the reaction by reading the samples at 240nm. Rates in mM/min were determined from 3 independent experiments performed in triplicate.

Creatine kinase assay

The EnzyChrom Creatine Kinase Assay Kit (BioAssay Systems) was applied to measure creatine kinase activity in mitochondria purified from wild type and parkin knock-out mouse brains following the manufacturer's instructions. Two brains were pooled each from 12 months old mice to provide the mitochondrial samples and normalized for total protein concentration. Mitochondria were incubated with 0 or 0.5mM H_2O_2 at room temperature for 20 minutes prior to assay. The creatine kinase-dependent catalytic conversion of creatine phosphate and ADP to creatine and ATP, was quantified indirectly by measuring NADPH at 340nm. The ATP produced by the reaction phosphorylates glucose to glucose-6-phosphate by hexokinase, which is then oxidized by NADP in the presence of glucose-6-phosphate dehydrogenase, yielding NADPH. Rates in mM/min were calculated for 3 independent experiments done in triplicate.

GSH and GSSG quantification - HPLC

Human and mouse brain pieces, and CHO cell pellets were homogenized in buffer containing 125 mM sucrose, 5 mM TRIS, 1.5 mM EDTA, 0.5%TFA and 0.5%MPA in mobile phase. Then samples were spun at 14000xg at 4 °C for 20 mins. Supernatants were collected and analyzed using an Agilent HPLC system equipped with a Pursuit

C₁₈ column (150 × 4.6 mm, 5 µm; Agilent Technologies) operating at a flow rate of 1 ml/min. The mobile phase consisted of 0.09% trifluoroacetic acid diluted in ddH₂O and mixed with HPLC-grade methanol in a 90:10 ratio. Standard solutions were used to estimate the retention times for GSH and GSSG. Using Agilent Chemstation software, absolute amounts of GSH and GSSG were acquired by integrating the area under the corresponding peaks, and values were calculated from standard curves.

GSH concentration determination by monochlorobimane assay

Stock solutions of assay dye (monochlorobimane (MCB), 22 mM) and glutathione-S-transferase (50 units/mL) were prepared in PBS and stored protected from light at -20°C. The working solution was prepared using 12.8 µL of stock MCB and 80 µL of stock glutathione-S-transferase in 4 mL PBS and stored on ice. Samples were prepared as follows: cells were lifted mechanically using cell-lifters, washed twice and re-suspended in ice-cold PBS, mixed by vortex and incubated on ice for 30 min. Following two freeze thaw cycles using solid CO₂, the samples were sonicated 1 min on wet ice (S220 Ultrasonicator from Covaris) and spun at 3000 × g, 4°C, for 5 min. Total protein concentration of supernatants was determined using Bradford assay. Samples and glutathione (GSH) standards (0- 13 µM) were plated in 25 µL aliquots in a 96-well plate with clear bottom and black sides. 25 µL of working solution was added to all experimental wells and protected from light for 15 min at room temperature. Fluorescence (ex 380 nm, em 461 nm) was measured using a Synergy H1Multi-Mode Plate Reader (Bio Tek). The amount of GSH detected in each sample was calculated using the regression curve obtained from the glutathione standards.

Tietze's enzymatic recycling determination of GSH and GSSG

The enzymatic recycling method described by Rahman et al.³⁹⁰ was used to determine reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in mouse brain lysates. Hemi-brains of wild type (n=3) and parkin KO (n=3) mice, at 13 and 11 months of age respectively, were collected, weighed and homogenized in 3X volume/weight of KPEX (0.1 M potassium phosphate, 5 mM EDTA, 0.1 % Triton X-100, 0.6 % sulfosalicylic acid, pH 7.5) using a glass Dounce homogenizer (50 passes). Samples were spun at 8000 x g, 4°C, for 5 min and the supernatant protein concentration was determined using Bradford assay. To determine the total glutathione (GSH + GSSG) concentration, the following stock solutions were freshly prepared in KPE (0.1 M potassium phosphate, 5 mM EDTA, pH 7.5): 5,5'-dithio-bis-[2-nitrobenzoic acid] (DNTB) at 0.6 mg/mL, nicotinamide adenine dinucleotide phosphate (NADPH) at 0.6 mg/mL and glutathione reductase at 3 units/mL. GSH standards were prepared in KPE at concentrations of 0-26 nM/mL. 20 µL of diluted sample or GSH standard was added per well and 120 µL of a 1:1 mixture of the DNTB and glutathione reductase stocks solutions was added to each assayed well. After 30 sec incubation, 60 µL of the NADPH was added and absorbance was immediately measured at 412 nm in 30 sec intervals for a total of 2 min. To determine the concentration of oxidized glutathione (GSSG), the samples were first diluted (1 in 4) in KPE and treated with 0.2 % 2-vinylpyridine for 1 h at room temperature. Excess vinyl-pyridine was quenched with 1 % triethanolamine and GSSG was measured using the same method as total glutathione except GSH standards were replaced with GSSG (0-26.24 nM/mL) treated with vinyl-pyridine and triethanolamine. The absolute values of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG)

per sample were calculated using the linear regression obtained from the change in absorbance/min plotted against the GSH or GSSG standard concentrations, respectively, and dividing by the total protein concentration. Absolute values for GSH were determined using the following equation: $GSH = [GSH + GSSG] - 2[GSSG]$ ³⁹⁰.

Parkin-mediated redox state recycling

Parkin protein buffer exchange to T200 protein buffer (50 mM Tris, 200 mM NaCl, pH 7.5) was first performed using repeat centrifugations (8 times 4000 x g at 4°C for 10 min) in Amicon Ultra 10 kDa MWCO filters. Protein concentration was adjusted to 10 µM using T200. Both reduced (GSH) and oxidized (GSSG) glutathione stocks were prepared in phosphate buffered saline at concentrations of 1 mg/mL (3250 µM) and 2.01 mg/mL (6560 µM) respectively. Glutathione standards of 0, 2.5, 5, 10 µM and 100 µM of both GSH and GSSG were prepared and combined in the following ratios to a final volume of 90 µL: 10 µM GSH: 0 µM GSSG, 9 µM GSH: 1 µM GSSG, 8 µM GSH: 2 µM GSSG, 6 µM GSH: 4 µM GSSG, 4 µM GSH: 6 µM GSSG, 2 µM GSH: 8 µM GSSG, 1 µM GSH: 9 µM GSSG, and 0 µM GSH: 10 µM GSSG. Recombinant-parkin (1 µL of a 10 µM solution) was added to the prepared mixtures and allowed to incubate at room temperature for 15 min. Samples were analyzed for GSH concentration using the monochlorobimane assay described above.

Glutathionylation assay

Glutathionylation of tagged and untagged parkin proteins was performed, as described previously³⁹¹. MBP-tagged parkin proteins were eluted from columns with excess

maltose. Concentrated eluates were supplemented with 0.1% DMSO (10 μ l DMSO in 10 ml PBS), and excess DTT and maltose were removed by several cycles of centrifugation with 30 kDa cut-off filters. Proteins/peptides (14 μ M) were incubated with 3 mM GSH for 1 h and then with 5 mM GSSG for 2 h at room temperature. Trypsin digestion was performed (Peptide: Trypsin = 20: 1) overnight at 4 °C. The Trypsin-digested fragments were run through MALDI analysis. To assay S-glutathionylation, the eosin-labeled GSSG (Di-E-GSSG) was used to glutathionylate proteins, as described previously ³⁸⁴. Di-E-GSSG has quenched fluorescence in the disulphide form, which increases ~20-fold upon reduction of its disulphide bond and formation of E-GSH. Blackened 96-well-plates were used in a PerkinElmer Victor3 multilabel counter containing a final well volume of 200 μ l in 0.1 M potassium phosphate buffer (pH 7.5), 1 mM EDTA. The reaction was started by addition of 20 μ M Di-E-GSSG to parkin proteins, followed by recording the fluorescence emission at 545 nm after excitation at 520 nm. Controls with no peptide added were used as fluorescent background. To confirm S-glutathionylation, reaction products were incubated with Di-E-GSSG. Aliquots of S-glutathionylated proteins were further treated with 10 mM DTT or with the complete GSH-Grx system. All samples were run on a non-reducing SDS-PAGE 4–12% acrylamide. The gel was exposed to UV transilluminator to visualize eosin-tagged glutathionylated protein. The same gels were later stained with Coomassie Blue staining. Di-Eosin-GSSG was purchased from IMCO, Sweden. Human Grx1, and Grx2 were prepared as described previously ³⁸⁴. Rat recombinant TrxR was a kind gift from Prof. Elias Arner.

Mass spectrometry analysis of glutathionylated parkin

The protein was treated with proteolytic digestion, using trypsin. The resulting peptides were separated using one dimension of liquid chromatography. The LC eluent was interfaced to a mass spectrometer using electrospray ionization and the peptides were analyzed by MS.

LC-MS/MS analyses were performed using an Easy-nLC chromatography system directly coupled online to a Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer with a Thermo Scientific™ Nanospray Flex™ ion source. The sample was injected from a cooled autosampler onto a 10 cm long fused silica tip column (SilicaTips, New Objective, USA) packed in-house with 1.9 μm C18-AQ ReproSil-Pur (Dr. Maisch, Germany). The chromatographic separation was achieved using an acetonitrile (ACN)/ water solvent system containing 0,1% FA and a gradient of 60 min from 5 to 35% of ACN. The flow rate during the gradient was 300 nL/ min.

MS/MS data were extracted and searched against in-house Mascot Server, (Revision 2.5.0), search engine that uses mass spectrometry data to identify and characterize proteins from sequence databases. The following parameters were used: trypsin digestion with a maximum of two missed cleavages; Carbamidomethyl (C), Oxidation (M), Deamidated (NQ) and Glutathione (G) as variable modifications; and a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.02 Da. The identified protein was filtered using 1% false discovery rate (FDR) and at least two peptides per protein as limiting parameters.

Protein carbonyl assay

Protein carbonyl colorimetric assay kit (Cayman chemical 10005020) was used to assay the carbonyl content in human and mouse brains or hearts. Pre-weighed tissues were rinsed in PBS and then homogenized in 1 mL cold PBS at pH 6.7 supplemented with 1 mM EDTA, using a Dounce homogenizer on ice. Homogenates were centrifuged at 10,000xg for 15 minutes at 4 °C. 200 µL of the supernatant was added to a tube with 800 µL 2,4-Dinitrophenylhydrazine (DNPH; sample tube) and 200 µL of the supernatant was added to a tube with 800 µL 2.5 M HCl (control tube), both tubes were incubated in the dark for 1 hour with occasional vortex. 1 mL 20 % TCA followed by 1 mL 10% TCA solutions were added to the centrifuged (10,000xg 10 minutes at 4 °C) pellet after discarding the supernatant. The resulting pellet was resuspended in 1 mL of 1:1 ethanol: ethyl acetate mixture and centrifuged 3 times to extract protein pellets. The final pellets were suspended in 500 µL guanidine hydrochloride and centrifuged. A total of 220 µL per sample and control supernatants were added to two wells of a 96-well plate, and the absorbance was measured at 360 nm. The corrected absorbance (CA, sample – control) was used in the following equation to obtain the protein carbonyl concentration: Protein Carbonyl (nmol/mL) = [(CA)/(0.011 µM⁻¹)](500 µL/200 µL). Total protein concentration from the sample tissues were measured to obtain the carbonyl content: protein carbonyl/total protein concentration.

MnSOD activity assay

Superoxide dismutase assay kit (Cayman chemical 706002) was used to assay MnSOD activity in mouse brains. Pre-weighed perfused mouse brain pieces were homogenized in

5 mL cold 20 mM HEPES buffer, pH 7.2, supplemented with EGTA, mannitol and sucrose, with a Dounce homogenizer on ice. The homogenates were centrifuged at 1,500xg 5 minutes at 4 °C. The SOD standards were prepared by adding 200 µL of the radical detector and 10 µL of the provided standards, in duplicates in a 96-well plate. The same was repeated for the samples. The reaction was initiated by adding 20 µL of xanthine oxidase to all the wells. Background absorbance was assayed by adding 20 µL xanthine oxidase to sample buffer (optional). The plate was incubated on a shaker for 30 minutes at room temperature. The absorbance was measure at 450 nm. The linearized SOD standard curve was plotted and used to calculate the MnSOD activity (U/mL) from the averaged sample absorbances.

Total RNA isolation, cDNA synthesis and PCR amplification

Pre-weighed cortex pieces from mouse brains were homogenized in QIAzol (Qiagen), at 1ml volume per 100mg of tissue, and incubated at room temperature for 5 minutes. 0.2mL of chloroform (per 1mL QIAzol) was added and the homogenates were shaken vigorously for 15 seconds, followed by a 2-3 minutes incubation at room temperature, the tubes were centrifuged at 12,000xg for 15 minutes at 4°C. The upper clear aqueous layer was transferred to a new tube and 1 volume of 70% ethanol was added, and mixed by vortexing. The solution was then added to an RNAeasy Mini spin column (Qiagen) placed in a 2mL collection tube and centrifuged for 15 sec at 8000xg at room temperature. The flow-through was discarded and 700 µL Buffer RW1 was added to the spin column and spun for 15 sec at 8,000xg. The same step was repeated with 500 µL Buffer RPE, one spin for 15 sec and a second spin for 2 min. An optional spin in a new

collection tube at full speed for 1 min to remove excess buffer was added. The RNAeasy Mini spin column was placed in a new collection tube, 50 µL RNase-free water was added directly to the membrane and centrifuged for 1 minute at 8000xg. A NanoDrop machine was used to measure the amount of total RNA obtained from the cortices. Turbo DNA-freeTM (Life Technologies) was used to remove trace to moderate amounts of contaminating DNA. SuperScriptTM IV First-Strand Synthesis System (Invitrogen) was used for cDNA synthesis reaction. iTaqTM Universal SYBR® Green Supermix (BIO-RAD) and select primer sets were used for PCR amplification of the newly synthesized cDNA templates, and controls, and analyzed by agarose gel electrophoresis and ethidium bromide staining. The following primers were used³⁹²⁻³⁹⁴:

DJ-1, F: ATCTGAGTCGCCTATGGTGAAG; R: ACCTACTTCGTGAGCCAACAG

GCLC, F: ATGTGGACACCCGATGCAGTATT; R: TGTCTTGCTTGTAGTCAGGATGGTTT

GCLM, F: GCCACCAGATTGACTGCCTTT; R: CAGGGATGCTTTCTTGAAGAGCTT

Actin, F: CTCCTCCCTGGAGAAGAGC; R: AAGGAAGGCTGGAAAAGAGC

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Differences between two groups were assessed using a Student t-test. Differences among 3 or more groups were assessed using a 1way or 2way ANOVA followed by Tukey, Dunnett or Bonferroni post hoc corrections (as indicated) to identify statistical significance. Subsequent post hoc tests are depicted graphically and show significance between treatments. For all statistical analysis

a cut-off for significance was set at 0.05. Data is displayed with p values represented as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

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Additional Information:

Data and materials availability: Original data associated with this study are available in the main text and extended data figures and tables; additional data will be made available upon request.

Supplementary Information is available for this manuscript.

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**Chapter 4: Manuscript 3 - Validation of an Anti-oxidant
Function by Parkin in Mammalian Brain Through the
Characterization of a Bi-genic *prkn*^{-/-}//*Sod2*^{+/-} Mouse Model**

Validation of an Anti-oxidant Function by Parkin in Mammalian Brain Through the Characterization of a Bi-genic *prkn*^{-/-}//*Sod2*^{+/-} Mouse Model

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Abstract

Parkin-linked Parkinson disease represents an early-onset variant of the disease, characterized by motor deficits in the absence of cognitive decline. The field currently lacks an animal model that accurately recapitulates mutant *PRKN*-linked parkinsonism. We recently found that parkin is a redox sensitive protein that maintains redox homeostasis by mediating several redox reactions that lower oxidative stress. We hypothesized that in human brain, parkin's absence causes the cumulative rise in oxidative radicals and subsequent death of dopamine neurons. We sought to test this in mice. To increase oxidative stress *in vivo*, we created a bi-genic mouse model that combines the loss of *prkn* with haploinsufficiency at the *Sod2* locus. We performed an age-dependent behavioural, neuropathological and biochemical characterization of *prkn*^{-/-}//*Sod2*^{+/-} mice. We found that the bi-genic mouse showed higher levels of hydrogen peroxide and nitrotyrosination in high energy-demanding tissues such as brain and heart, as expected. However, even at 12 months of age, *prkn*^{-/-}//*Sod2*^{+/-} mice did not show any motor deficits in a battery of behavioural tests when compared to age-matched wild-type (WT) and single gene-mutant littermates. Neuropathologically, there was no genotype-dependent reduction in the number of tyrosine hydroxylase-expressing neurons in the *S. nigra* at this age. These results support an *in vivo* role for parkin to regulate oxidative stress levels; however, at 12 months of age, its absence did not lead to detectable evidence of parkinsonism at the motor or neuropathological levels.

Introduction

Parkinson disease (PD) is an incurable neurodegenerative disorder, manifesting as motor deficits due to the progressive loss of dopaminergic neurons in the *Substantia nigra pars compacta* (SNc). Loss-of-function mutations in the *PRKN* gene were the first mutations to be causally linked to recessive forms of PD¹⁹³. Patients with parkin-linked parkinsonism, also referred to as autosomal recessive PD (ARPD), or early-onset PD (EOPD), develop a variant of parkinsonism. They develop the disease with an earlier age of onset and present with only motor deficits unlike typical PD, which is associated with cognitive deficits as the disease progresses. Neuropathologically, they do not have evidence of Lewy body pathology in surviving neurons and cell loss is restricted to the SNc and *Locus coeruleus* (LC)^{199,208,209,395}.

Several parkin-linked genetic mouse models have been created and characterized to study early onset parkinsonism^{94,95,299-302,396,397}. All of these models failed to recapitulate the disease as seen in humans. The mouse models do not develop motor deficits nor nigral degeneration, including in a triple knockout mouse of three recessively inherited PD genes (*prkn*, *DJ-1* and *PINK1*)³³¹. Nevertheless, *prkn*-deficient mice developed signs of chronic oxidative stress, including higher levels of protein carbonylation and upregulation of redox proteins and enzymes^{94,95}.

Parkin is an E3-ubiquitin ligase and is involved in regulating mitophagy together with PINK1^{150,268,323}. If these functions underlie the selective protection conferred by parkin to dopamine neurons in human brain remains unknown. We recently characterized a new function of parkin in maintaining redox homeostasis and mediating redox reactions to

lower chronic oxidative stress in mammalian brains^{349,373}. We propose that this function contributes to the neuroprotection conferred by parkin. We sought to test this in vivo with the creation of a bi-genic model that combines genetically-encoded increased oxidative stress, with parkin deficiency.

Sod2 encodes manganese superoxide dismutase (MnSOD) that is the first line of defense against a rise in reactive oxygen species (ROS), namely superoxide anion (O_2^-), in the mitochondria. A loss of *SOD2* has been implicated in mitochondrial dysfunction and the pathogenesis of several neurodegenerative disease, including PD³⁹⁸. *Sod2*-null mouse models are neonatal lethal and present with endogenous mitochondrial oxidative stress, motor deficits and ultimately neurodegeneration^{377,399}. Mice deficient for parkin, DJ-1, and only haploinsufficient at the *Sod2* locus (homozygous mutants are lethal), develop behavioural and neurotransmitter abnormalities⁴⁰⁰.

Based on parkin's role in mediating redox homeostasis and MnSOD's involvement in the defence against mitochondrial ROS generation, we hypothesized that the genetic combination of *prkn* deficiency and *Sod2* haploinsufficiency would generate a mouse model with a higher oxidative stress burden leading to behavioural deficits and nigral degeneration. Published parkin-null mouse models did not exhibit a parkinsonian phenotype, namely motor deficits and nigral cell loss, when compared to wild-type (WT) littermates. Additionally *Sod2*^{+/-} mice have not yet been fully characterized behaviourally and biochemically, with the exception of *Sod2*-null mice presenting with decreased survival and increased oxidative stress. Hence, we believed that the addition of a parkin

deficiency to a *Sod2* haploinsufficiency would generate a stronger oxidative stress phenotype. Together, our results demonstrated increased levels of endogenous H₂O₂ in *Sod2*^{+/-} mouse brains, and the addition of a loss of function of parkin in the bi-genic mouse model (*prkn*^{-/-} // *Sod2*^{+/-}) further increased signs of oxidative stress, seen as higher levels of ROS, protein nitrotyrosination levels, and protein carbonyls in high energy-demanding tissues such as brain and heart, as expected. By 12 months of age however, there was no dopaminergic cell loss nor gross motor deficits.

Results

Generation of the *prkn*^{-/-}//*Sod2*^{+/-} bi-genic mouse

The bi-genic mouse was generated by first crossing *prkn*^{-/-} mice³⁰⁰ with *Sod2*^{+/-} mice³⁹⁹(**Fig. 4.1a**); both were maintained on a pure C57Bl/6J background. *prkn* and *Sod2* genes are both located on mouse chromosome 17, separated by 1 centimorgan (cM). This equates to a 1% recombination rate (**Fig. 4.1b**), which signifies a one percent chance that *prkn* on chromosome 17 will become separated from *Sod2*, on the same chromosome due to crossing over in a single generation⁴⁰¹. Heterozygous offspring at both the *prkn* and *Sod2* loci, were interbred over 10 generations to allow for recombination and produce the desired dual genetic hit *prkn*^{-/-}//*Sod2*^{+/-} (**Fig. 4.1a, c**). The bi-genic mouse model has no detectable parkin protein, reduced MnSOD levels and activity³⁴⁹. Two experimental cohorts of WT, *prkn*^{-/-}, *Sod2*^{+/-}, and *prkn*^{-/-}//*Sod2*^{+/-} were bred and aged to 6 months and 12 months respectively and used for behavioural and biochemical analyses (**Extended Data Table 4.1 and Extended Data Fig. 4.1**). A backup cohort was maintained in parallel. Group sizes and equal sex ratios within genotypes were comparable within and across all cohorts (**Extended Data Table 4.1**).

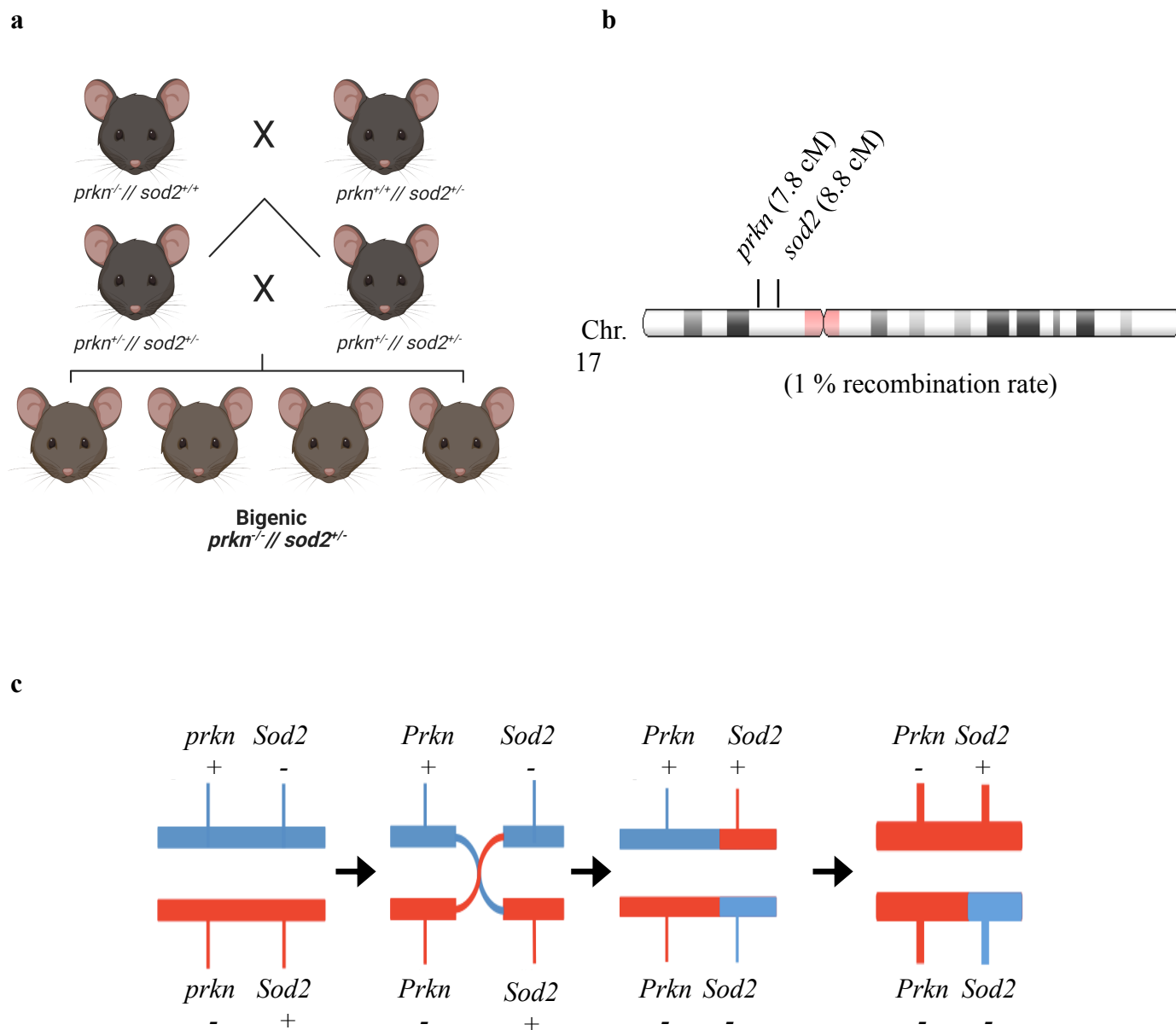
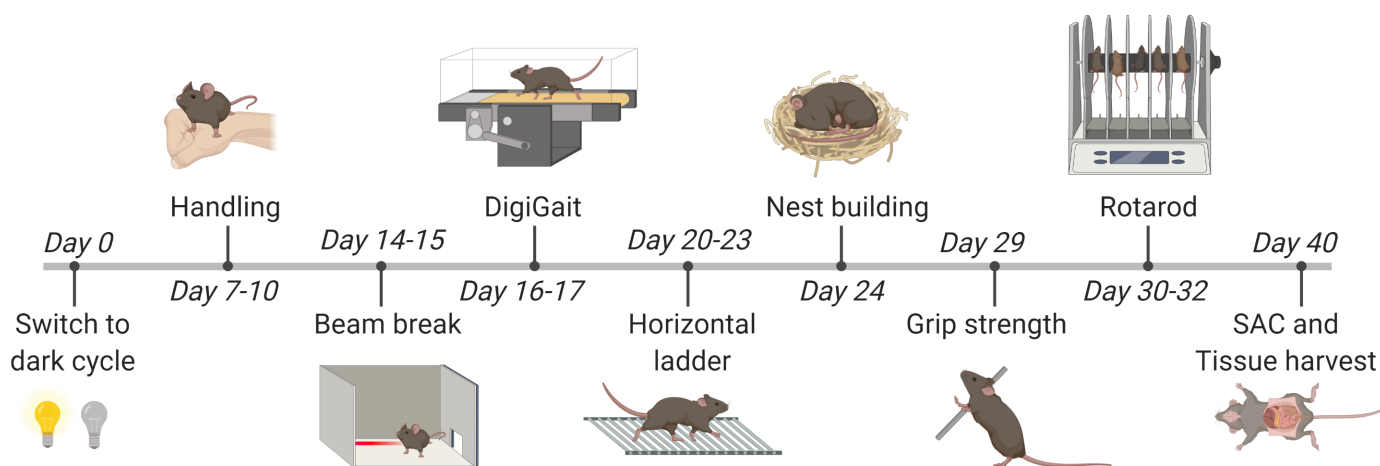


Figure 4.1: Generation of the *prkn*^{-/-} // *Sod2*^{+/-} bi-genic mouse.

a, Graphical depiction of the crossing between *prkn*^{-/-} and *Sod2*^{+/-} mice to generate the new bi-genic (*prkn*^{-/-} // *Sod2*^{+/-}) mouse model.

b, Schematic representation of mouse chromosome 17, with *prkn* and *Sod2* loci separated by 1 centimorgan (cM).

c, Graphical depiction of the recombination between the *prkn* and *Sod2* alleles, at a rate of 1%, to produce the desired crossing.



Extended Data Figure 4.1: Experimental Timeline

Graphical depiction of the timeline of the behavioural tests planned for the 6 mths-old and 12 mths-old cohorts; starting with the switch to the dark cycle and ending with animal sacrifice and tissue harvest. Created with Biorender.com.

6 months old cohort			
	M	F	Total
WT	4	5	9
<i>prkn</i> ^{-/-}	7	5	12
<i>Sod2</i> ^{+/-}	5	5	10
Bigenic	8	4	12
Total	24	19	43
6 months old repeat cohort			
	M	F	Total
WT	5	5	10
<i>prkn</i> ^{-/-}	5	5	10
<i>Sod2</i> ^{+/-}	5	6	11
Bigenic	7	5	12
Total	22	21	43
12 months old cohort			
	M	F	Total
WT	5	5	10
<i>prkn</i> ^{-/-}	4	5	9
<i>Sod2</i> ^{+/-}	4	6	10
Bigenic	5	5	10
Total	18	21	39

Extended Data Table 4.1: Behaviour mouse cohorts.

Summary of our 6 mths-old and 12 mths-old behaviour cohorts. Mouse numbers per genotype, wild-type (WT); *prkn*-null (*prkn*^{-/-}); *Sod2* haploinsufficient (*Sod2*^{+/-}); and bi-genic (*prkn*^{-/-} // *Sod2*^{+/-}) are displayed, along with the female to male ratios.

The bi-genic mouse displays signs of elevated oxidative stress in the absence of nigral degeneration

We previously reported a cytosol-specific increase in H₂O₂ levels in brains of bi-genic mice when compared to *prkn*^{-/-} and *Sod2*^{+/-}, at 2-4 months of age, with no changes in mitochondrial H₂O₂ levels³⁴⁹. Here we assessed similar readouts at 6 months of age. The bi-genic mouse exhibited higher levels of endogenous H₂O₂ in heart tissues when compared to WT littermates (p<0.05; **Fig. 4.2a**). In the cortex, the bi-genic mouse showed higher H₂O₂ levels when compared to WT, *prkn*^{-/-} and *Sod2*^{+/-} mice (p<0.05; **Fig. 4.2b**). In midbrain tissue, the bi-genic mouse had higher ROS levels, namely H₂O₂, when compared to WT mice (p<0.05; **Fig. 4.2c**). We also quantified the more reactive superoxide anion (O₂⁻) levels in hemibrains from the 6 months old cohort by HPLC. There, the bi-genic mouse showed significantly lower levels (p<0.01; **Fig. 4.2d**) compared to age-matched WT mice. When quantifying nitrotyrosination profiles resulting from increased levels of superoxide and consequently peroxynitrite, *prkn*^{-/-} and bi-genic mice display significantly higher levels when compared to WT littermates in the heart (**Fig. 4.2e, f**). The trend is only significantly higher in *prkn*^{-/-} midbrains, when compared to WT mice (**Fig. 4.2g, h**). Additionally, the bi-genic mouse exhibited significantly higher levels of brain protein carbonyls, when compared to WT, *prkn*^{-/-} and *Sod2*^{+/-} mice³⁴⁹.

Given the variability in irreversible oxidative stress markers, namely nitrotyrosination profiles, in the brain of the bi-genic mouse when compared to WT and *prkn*^{-/-} mice (**Fig. 4.2g, h**), in addition to H₂O₂ levels in bi-genic mouse brains being only significantly

higher than WT mice (**Fig. 4.2c**), we sought to investigate whether the increase in brain H_2O_2 levels and the aging process would generate midbrain-specific cell loss. We used stereological methods to quantify the number of dopaminergic neurons in the SNc by counting tyrosine hydroxylase (TH)-positive neurons. At 12 months of age no genotypic differences in TH positive cell counts were observed (**Fig. 4.2i**).

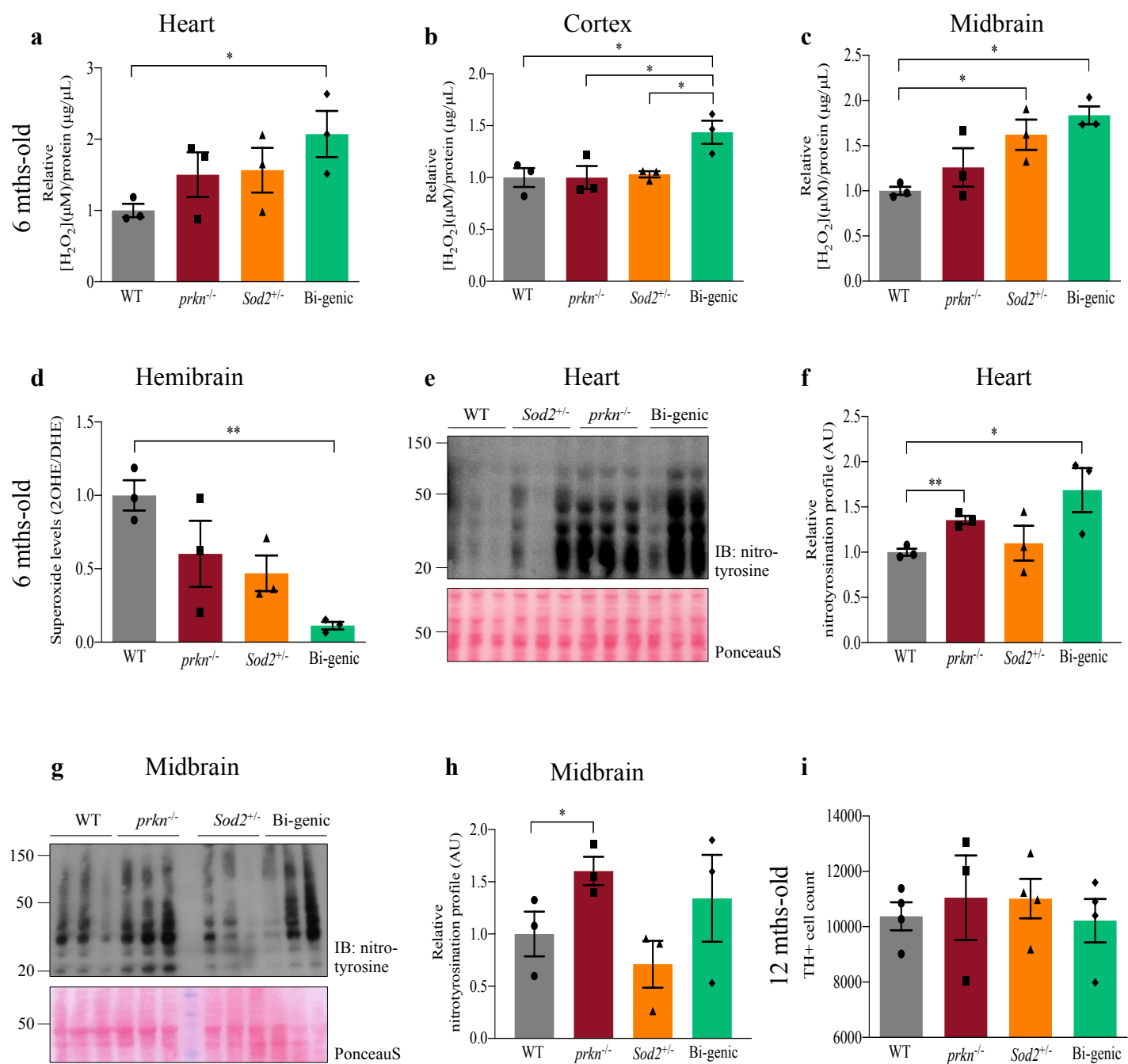


Figure 4.2: The bi-genic mouse displays signs of elevated oxidative stress in the absence of nigral degeneration.

Hydrogen peroxide (H_2O_2) levels measured in 6 mths-old **a**, hearts, **b**, cortex, and **c**, midbrains.

d, Superoxide (O_2^-) levels measured by HPLC as the ratio of 2-hydroxyethidium (2OHE) to dihydroethidium (DHE) in hemibrains of 6 mths-old mice.

e, Representative Western blot of nitrotyrosination in hearts of 6 mths-old mice.

f, Quantification of nitrotyrosination signals in 6 mths-old mouse hearts.

g, Representative Western blot of nitrotyrosination in midbrains of 6 mths-old mice.

h, Quantification of nitrotyrosination signals in 6 mths-old mouse midbrains.

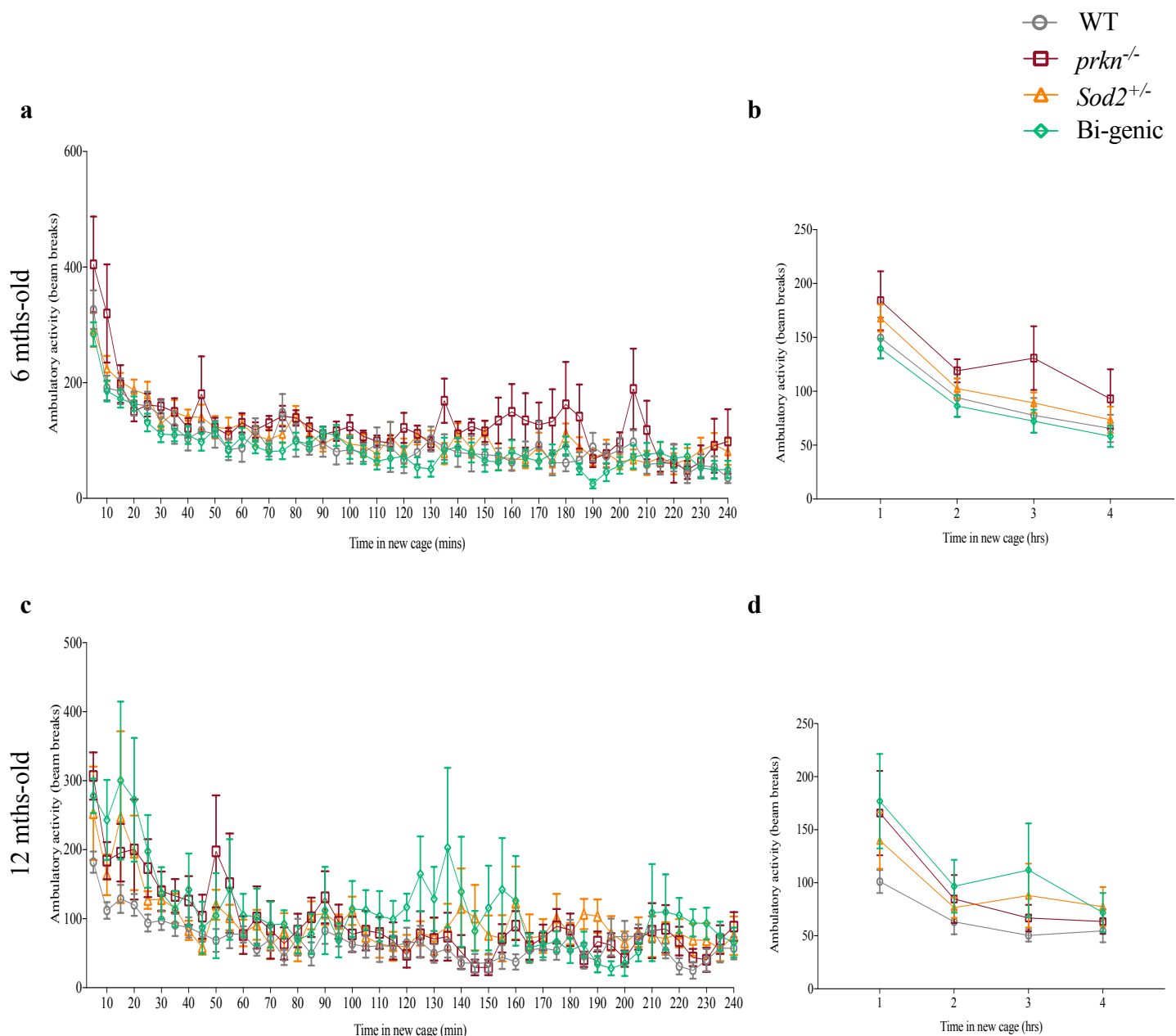
i, Tyrosine hydroxylase (TH) positive cell counts of 12 mths-old mouse brains.

A Student t-test or 1way ANOVA was used for statistical analysis (*= <0.05; and **= <0.01).

Prior to tissue harvest, mice were evaluated on an extensive battery of behavioural tests that have been validated to be sensitive to changes observed in parkinsonism models. We sought to observe if the expected increase in oxidative stress associated with altered behavioural outcomes.

The bi-genic mouse shows no differences in habituation and general locomotion

When placed in a novel cage and monitored for 4 hours in 5 minutes bins, using infrared light beams and sensors that monitor the animal's ambulatory activity, a 2way ANOVA analysis showed no differences at 6 and 12 months between the bi-genic mouse and its littermates (Wild-type, WT; *prkn*-null, *prkn*^{-/-}; and *Sod2* haploinsufficient, *Sod2*^{+/-}) in general locomotion. This was observed as the high activity and exploration of the novel cage over the first hour (**Fig. 4.3a, c**). Additionally, as expected, the animals habituated to their new environment, with no genotypic differences at 6 and 12 months of age (2way ANOVA analysis). This was observed as the decrease in activity over the last 3 hours of testing (**Fig. 4.3a, c**). The trend was similar when assessing each hour of the 4 hours testing time (**Fig. 4.3b, d**). This indicated no evidence of gross deficits in baseline mobility and exploratory activities across all 4 genotypes and with aging.



The bi-genic mouse displays no motor deficits on the rotarod

To evaluate motor coordination, balance, and learning⁴⁰², littermates were tested on the rotarod over four trials a day for three days. By utilizing an accelerating protocol (4-40 revolution per minute (rpm) over 5 minutes) as opposed to a constant speed protocol, we minimized fatigue and provided a more challenging paradigm to detect finer coordination deficits, as previously described⁴⁰³. As expected, WT mice displayed task learning, which was observed by the increasing in the latencies to fall over the 3 days of testing (**Fig. 4.4a, b**), as well as across the 4 trials per day (data not shown), at 6 and 12 months of age. This confirmed that the behaviour test performed as planned. 2way ANOVA analysis showed no differences in motor coordination, measured as latencies to fall off the accelerating rod across the 4 genotypes at both 6 and 12 months of age, the (**Fig. 4.4a, b**). It is important to note that *prkn*^{-/-}, *Sod2*^{+/-}, and bi-genic mice also showed task learning capacities comparable to WT animals, and overall across the 4 genotypes the latencies to fall decreased with age, as expected.

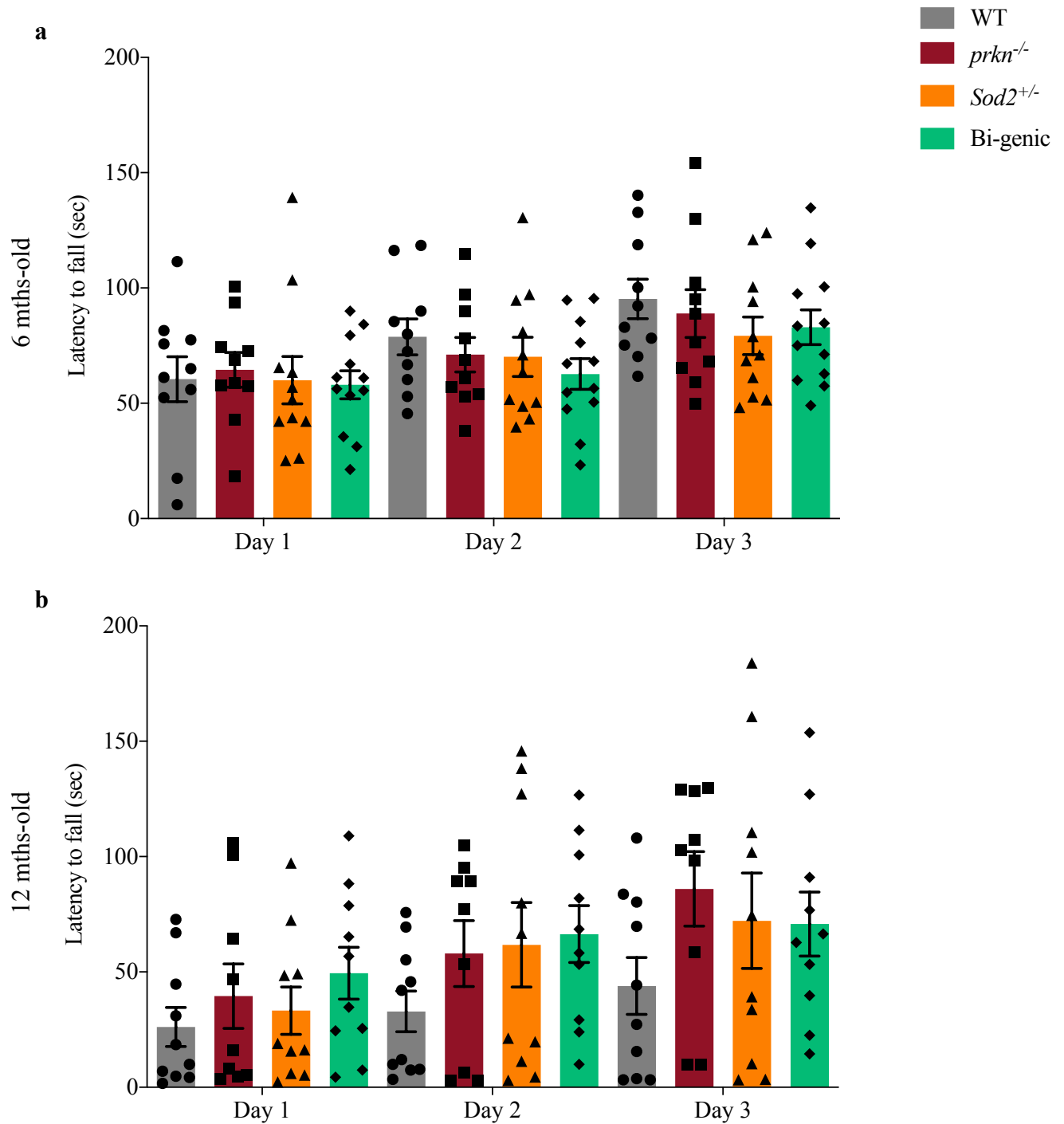


Figure 4.4: The bi-genic mouse displays no motor deficits on the rotarod.

a, The average latency to fall in seconds (sec) over 4 trials of the 6 mths-old cohort. The 3 days of trials are represented.

b, The average latency to fall in seconds (sec) over 4 trials of the 12 mths-old cohort. The 3 days of trials are represented.

A 2way ANOVA was used for statistical analysis.

The bi-genic mouse exhibits no deficits on the horizontal ladder test

To evaluate gait and coordination, a skilled walking test was used. The mice were first trained to cross a horizontal ladder with uneven rungs that were encased in two plexiglass walls on each side. The home cage was placed on one end as incentive to cross the ladder. On testing day, the mice were recorded crossing the ladder over 4-5 trials. The number of correct steps and errors (missed rung or cheated by placing the paw on the plexiglass wall) were quantified⁴⁰⁴(**Fig. 4.5a**), and 1way ANOVA analyzed. When assessing hindlimb errors per step and percent hindlimb errors, the bi-genic mouse displayed no deficits at 6 and 12 months of age, when compared to WT, *prkn*^{-/-}, and *Sod2*^{+/-} mice (**Fig. 4.5b, c, e, f**). As a gross analysis of gait and to evaluate whether the bi-genic mouse took more or fewer steps to cross the ladder, the average hindlimb step count was quantified. There were no genotypic differences at both ages (**Fig. 4.5d, g**). Similar outcomes were observed in the forelimb of both cohorts (data not shown). As expected, the number and percent of hindlimb errors increased with age across all 4 genotypes, while the average step count remained the same. Additionally, observing no differences in the average hindlimb step count confirmed that *prkn*^{-/-}, *Sod2*^{+/-}, and bi-genic mice were able to perform the task as planned and comparable to WT age-matched animals.

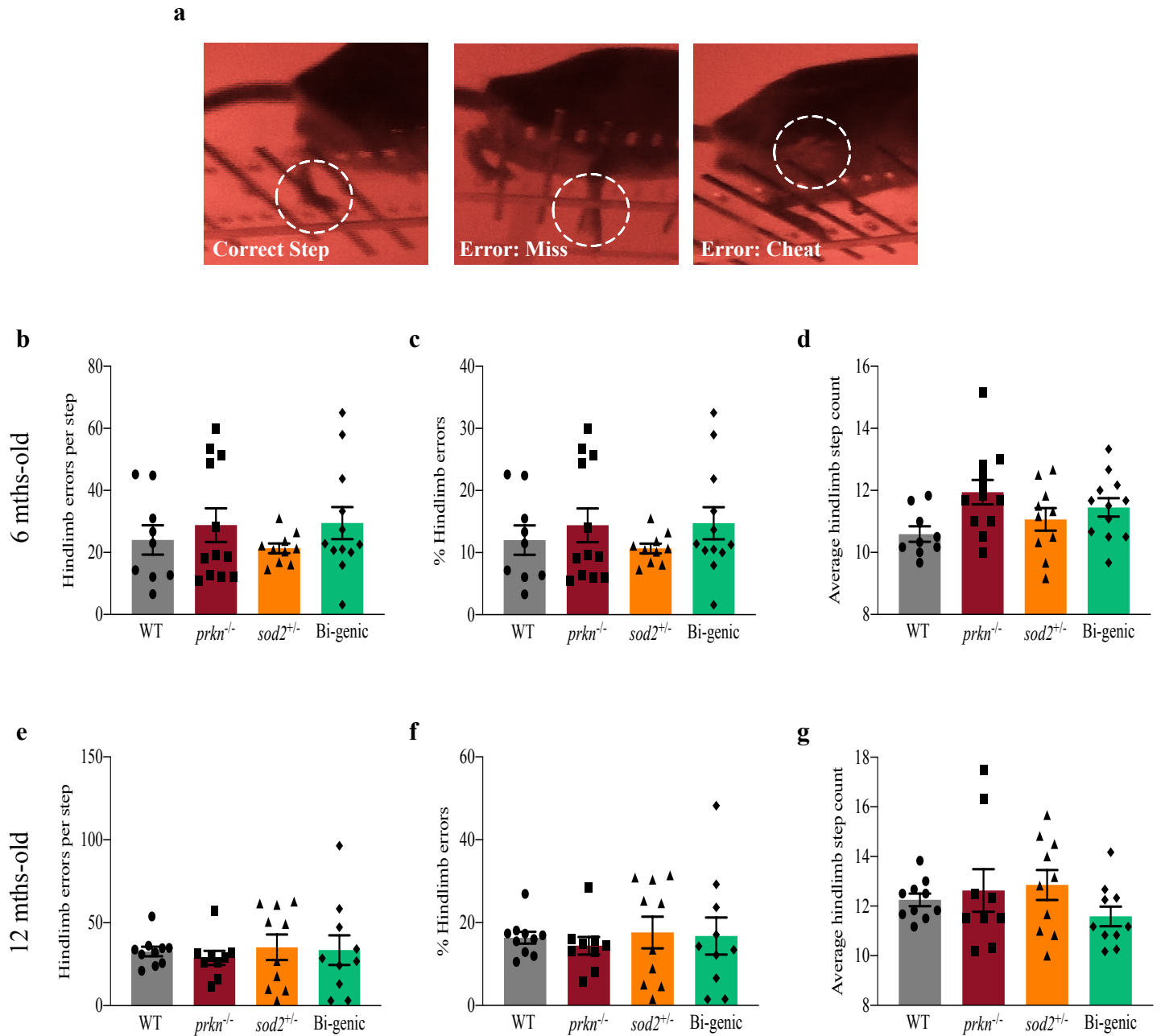


Figure 4.5: The bi-genic mouse exhibits no deficits on the horizontal ladder test.

a, Representative images of a correct step and 2 errors; a miss and a cheat.

b, The number of hindlimb errors per step of the 6 mths-old cohort.

c, The percentage of hindlimb errors of the 6 mths-old cohort.

d, The average hindlimb step count of the 6 mths-old cohort.

e, The number of hindlimb errors per step of the 12 mths-old cohort.

f, The percentage of hindlimb errors of the 12 mths-old cohort.

g, The average hindlimb step count of the 12 mths-old cohort.

A 1way ANOVA was used for statistical analysis.

The bi-genic mouse displays no gait or posture deficits on the DigiGait analysis

The DigiGait imaging system is a ventral plane videography instrumentation that allows for gait and posture analysis by continuously imaging the underside of mice while they walk on a treadmill with speed varying from 10 to 18cm/s. Built-in algorithms can quantify several readouts to assess dynamic gait signals. This technique has been used in recent studies to better evaluate subtle changes in gait and locomotion (observed in PD patients), indiscernible with other behavioural tests, in several animal models of PD⁴⁰⁵⁻⁴⁰⁷. Confirming the validity of the test, WT animals behaved and scored as expected, and as previously published⁴⁰⁵⁻⁴⁰⁷. However, a 2way ANOVA analysis at 6 and 12 months of age showed no quantifiable differences in forelimb and hindlimb stride lengths (cm) across all four genotypes (**Fig. 4.6a, b, e, f**). When evaluating stance widths (cm) and step angles (°), there were also no genotypic differences in both cohorts (**Fig. 4.6c, d, g, h**, 2way ANOVA analysis). Gait measures were not altered with age across all 4 genotypes.

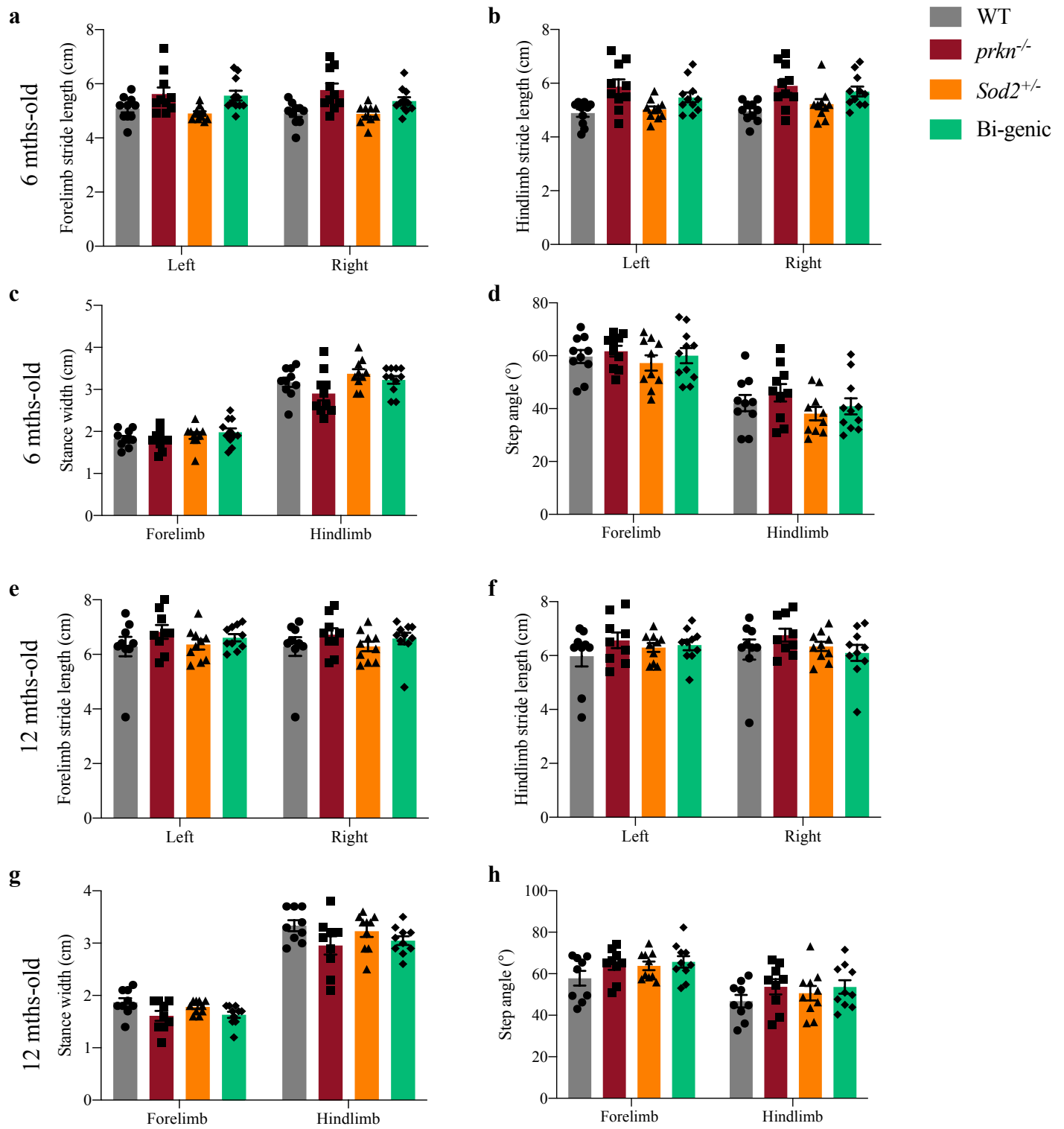


Figure 4.6: The bi-genic mouse displays no gait or posture deficits on the DigiGait analysis.

a, Forelimb, and **b**, hindlimb stride length in centimeters (cm) of the 6 mths-old cohort.

c, Stance width (cm), and **d**, step angle in degrees (°) of the 6 mths-old cohort.

e, Forelimb, and **f**, hindlimb stride length of the 12 mths-old cohort.

g, Stance width, and **h**, step angle of the 12 mths-old cohort.

A 2way ANOVA was used for statistical analysis.

The bi-genic mouse displays similar grip strengths as its littermates

When behaviourally evaluating PD animal models, grip strength is not routinely used, as it is not one of the cardinal motor symptoms observed in PD patients. Nevertheless, in a recent report, it is suggested that early-onset reduced grip strength, observed as low muscle strength in late adolescence, could predict the development of PD⁴⁰⁸. Additionally, as it relates to our model, parkin has been observed in high expression in skeletal muscles¹⁹³. Therefore, we analyzed grip strength in WT, *prkn*^{-/-}, *Sod2*^{+/-}, and *prkn*^{-/-}//*Sod2*^{+/-} mice. The forelimb grip strength was measured, in kilogram-force (kgF), using a force transducer that recorded the maximum force generated by the animal as it was pulled off of a grate. There were no genotypic differences in the average forelimb grip strength across 5 trials at 6 and 12 months of age (**Fig. 4.7a,e**), when analyzed by 1way ANOVA. When assessing only the maximum forelimb grip strength, there were also no genotypic differences at both ages (**Fig. 4.7b,f**). To determine if body weight is a contributing factor, the average and maximum forelimb grip strengths were normalized to body weight. After normalization, no differences between the bi-genic mouse and WT, *prkn*^{-/-}, and *Sod2*^{+/-} mice, at 6 months and 12 months of age remained. (**Fig. 4.7c, d, g, h**). Grip strength measures did not decrease with age across all 4 genotypes.

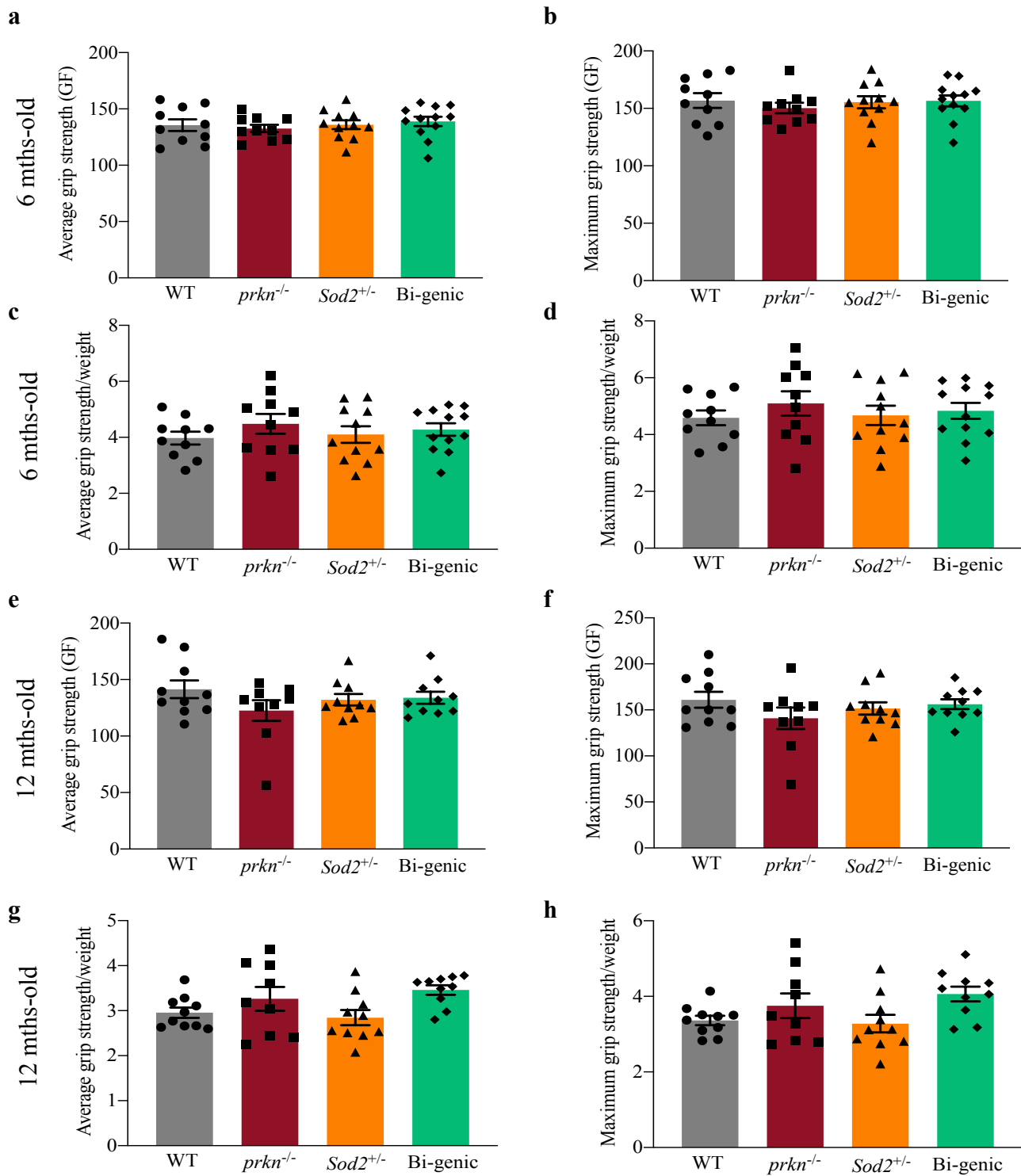


Figure 4.7: The bi-genic mouse displays similar grip strengths as its littermates.

a, Average grip strength of the 6 mths-old cohort. **b**, Maximum grip strength of the 6 mths-old cohort. **c**, Average grip strength per weight of the 6 mths-old cohort. **d**, Maximum grip strength per weight of the 6 mths-old cohort.

e, Average grip strength of the 12 mths-old cohort. **f**, Maximum grip strength of the 12 mths-old cohort.

g, Average grip strength per weight of the 12 mths-old cohort. **h**, Maximum grip strength per weight of the 12 mths-old cohort.

A 1way ANOVA was used for statistical analysis.

The bi-genic mouse presents with no deficits in nest building

Nest building assesses a complex and innate activity in mice, including motivation and fine motor skills. It can be qualitatively analysed⁴⁰⁹ (**Fig. 4.8a**) and can be impaired in Parkinson disease models⁴¹⁰. The bi-genic mouse did not score significantly lower than WT, *prkn*^{-/-}, and *Sod2*^{+/-} mice at 6 and 12 months of age, when the scoring (1-5) was averaged, and analyzed by 1way ANOVA on ranks with a non-parametric Kruskal-Wallis test (**Fig. 4.8b, d**). When assessing the frequencies of each score, a 2way ANOVA analysis showed that the bi-genic mouse did not significantly score lower (scores 1-3) at 6 and 12 months of age, when compared to WT, *prkn*^{-/-}, and *Sod2*^{+/-} mice (**Fig. 4.8c, e**).

Each cohort contained an equal distribution of males and females. When analyzing the battery of behavioural tests, no sex effects were observed (data not shown). Additionally, the body weight of each animal was recorded, and it was not a contributing factor to the lack of differences noted across all behavioural tests and genotypes (data not shown). Lastly, a third cohort was bred in parallel and aged to 6 months to repeat the rotarod test. The “repeat” cohort performed similarly on the battery of behavioural test as the “original” 6 months old cohort.

Discussion

The goal of our study was to generate a model for oxidative stress mediated-recessive parkinsonism (**Fig. 4.1**), based on our findings that parkin protected against excessive oxidative stress *in vivo*. To this end, we generated a mouse model which combined genetically encoded chronic oxidative stress due to haploinsufficiency at the *Sod2* locus, and we compared these mice to those that also lacked parkin. Age-dependent analysis of these mice revealed that indeed, the presence of parkin protein lowered oxidative stress in the brain and heart muscle (two high energy demanding organs; **Fig. 4.2a-h**); however, by 12 months of age, the mice did not exhibit motor deficits (**Fig. 4.3-4.8**) nor any underlying degeneration of their dopamine neurons in the SNc (**Fig. 4.2i**). Of note, we did not interrogate the striatum (terminal density nor neurotransmitter release, as it has been done in *prkn*^{-/-} mouse models³⁰⁰), which is warranted and which may be a more sensitive measure of early stages of degeneration, such as in the form of axonal retraction.

The dual genetic hit resulted in increased oxidative stress markers in mouse tissues, as expected^{349,377}. This rise was, however, insufficient to cause neurodegeneration in the first 12 months of age. Potential protective compensatory mechanisms could help explain this. Further investigation of related redox and anti-oxidant pathways revealed that there was an upregulation of reduced glutathione (GSH) levels in the bi-genic mice when compared to age-matched WT, *prkn*^{-/-} and *Sod2*^{+/-} mice³⁴⁹. GSH is the most abundant cytosolic low molecular weight thiol involved in antioxidant defense and regulating metabolism⁴¹¹. Additionally, GSH deficiency contributes to oxidative stress and plays a

role in aging and the pathogenesis of many diseases, including PD³⁴³. In the absence of parkin, mice upregulated GSH, presumably as a compensatory mechanism to keep ROS levels controlled and maintain redox homeostasis. This would be consistent with the lack of increase in endogenous H₂O₂ levels in *prkn*^{-/-} brains when compared to WT^{76,300,349}.

Another mechanism that could underlie the lack of a strong phenotype relates to mitochondria-generated compensatory mechanisms associated with the decrease in MnSOD activity in the bi-genic mouse³⁴⁹. In response to increasing levels of superoxide, a more efficient VDAC-mediated shuttling of the O₂⁻ radical into the cytosol occurs, where it is subsequently reduced by *sod1*³⁷⁸. The unexpected decrease in O₂⁻ levels in the bi-genic mouse brain (**Fig. 4.2d**), may reflect such a compensatory mechanism. The bi-genic mouse essentially models a mitochondrial hit (*Sod2* haploinsufficiency) with the addition of a cytosolic hit (loss of function of parkin). The presence of both cytosolic and mitochondrial compensation may also explain the lack of a strong phenotype, including of synergistically elevated nitrotyrosination of the brain in the bi-genic mouse, in contrast to our expectation (**Fig. 4.2g, h**).

If true, and based on the aforementioned observations, then inhibition of cytosolic GSH pathway in addition to parkin deficiency, should pose a greater parkin deficiency-linked threat to at-risk neurons. This could be accomplished through the disruption of the mouse glutamate-cysteine ligase modifier subunit (*Gclm*) gene, coding for the rate-limiting enzyme in the GSH-biosynthesis pathway⁴¹², or treatment with buthionine sulfoximine (BSO)⁴¹³.

We speculate that the lifespan of mice, the nature of oxidative radicals generated, and degree of stress, or any combination thereof, together with underlying compensatory mechanisms may have been insufficient to lead to nigral cell loss in these bi-genic mice. In comparison to human brain, there is another possible difference that makes parkin critical for the survival of dopaminergic neurons in the human brain, but not the mouse. That relates to the metabolism of dopamine. In addition to reducing H₂O₂ levels we found that parkin conjugated dopamine metabolites, in a concentration-dependent manner, and augmented neuromelanin formation in vitro³⁷³. Additionally, it prevented dopamine-mediated rise in oxidative stress and cell death; both of which are specific feature hallmarks of PD-patients' SNc³⁷³. Neuromelanin deposits are specific to humans and primates, and are not observed in aging mouse brains, which could, yet again, explain the lack of induction of PD pathogenesis in mouse models. In addition, dopamine metabolism has been reported to induced several pathway of oxidative stress⁵³. Therefore, a possible way to drive neurodegeneration in mice, and in particular models with increased oxidative stress (*prkn*^{-/-}, *Sod2*^{+/-}, and bi-genic) would be to include the additional hit of dopamine toxicity through L-dopa administration or monoamine oxidase (MAO)-B inhibition^{414,415}. Furthermore, research on neuromelanin production in mice, using tyrosinase overexpression, to produce neuromelanin and features of parkinsonism, is underway³⁴⁶.

The impact of oxidative stress, including parkin-mediated redox imbalance, on the pathogenesis of PD has gained strong ground in recent years⁴¹⁶⁻⁴¹⁸. Here we created a mouse model of chronic oxidative stress in the absence of a parkinsonian phenotype.

Building refined models in the future by focusing on further altering redox homeostasis, in addition to parkin deficiency, such as by adding dopamine radical-mediated oxidative stress or altering GSH levels, should better recapitulate EOPD in rodents. This, to create more opportunities to develop disease-modifying therapies for early onset parkinsonism^{347,419,420}.

Materials And Methods

General Information

All animal-related experiments were conducted in accordance with the Canadian Council on Animal Care Standards and the Animals for Research Act and were approved by the University of Ottawa Animal Care Council. The mice were bred in parallel (WT and *Sod2*^{+/-} from a *prkn*^{+/+} // *Sod2*^{+/+} x *prkn*^{+/+} // *Sod2*^{+/-} crossing; and *prkn*^{-/-} and bi-genic from a *prkn*^{-/-} // *Sod2*^{+/+} x *prkn*^{-/-} // *Sod2*^{+/-} crossing) and group housed (3-5 mice per cage) on a reversed 12h dark: 12h light cycle with food and water available *ad libitum*. Room temperature was maintained at 23°C and humidity at 30-40%.

All behaviour testing was performed in the Behavioural Core Facility at the University of Ottawa. Mice were minimally disturbed during behavioural testing. Body weights were measured before testing began and after it was done; before animals were sacrificed and tissues harvested. Behaviour testing was conducted blinded to the genotype of the mice. Behaviour tests were separated by 2-4 days and all mice were brought into the dark testing room with red light to habituate for 30 minutes to an hour prior to testing. Mice were handled once per day for four days before testing began.

Mouse lines

Wild-type (WT) C57BL/6 mice were purchased from Jackson laboratory. The *prkn*^{-/-} mice (C57BL/6 background) were obtained from Dr. Brice's lab³⁰⁰. The *Sod2*^{+/-} mice (C57BL/6 background) were generated by Dr. Lebovitz³⁹⁹, and purchased from Jackson laboratory. *prkn*^{-/-} // *Sod2*^{+/+} and *Sod2*^{+/-} // *prkn*^{+/+} mice were crossed to generate *prkn*^{+/-}

//*Sod2*^{+/-} offsprings, that were then interbred to produced the desired *prkn*^{-/-} //*Sod2*^{+/-} bi-genic mouse.

Genotyping

Ear tissue was collected for genotyping. DNA was extracted from the tissue by incubating the ear sample in 1X solution A (Solution A (10X): 250mM NaOH, 2mM EDTA, in water) at 95°C for 30 minutes, followed by neutralizing the reaction with 1X solution B (Solution B (10X): 400mM Tris-HCl, in water). Standard polymerase chain reaction (PCR) was used to amplify the *prkn* and *Sod2* loci. The following primers were used:

prkn^{+/+}

Forward: 5' TGCTCTGGGGTTCGTC 3'

Reverse: 5' TCCACTGGCAGAGTAAATGT 3'

prkn^{-/-}

Forward: 5' TTGTTTTGCCAAGTTCTAAT 3'

Reverse: 5' TCCACTGGCAGAGTAAATGT 3'

Sod2^{+/+}

Forward: 5' TGAACCAGTTGTGTTGTCAGG 3'

Reverse: 5' TCCATCACTGGTCACTAGCC 3'

Sod2^{-/-}

Forward: 5' TGTTCTCCTCTTCCTCATCTCC 3'

Reverse: 5' ACCCTTTCCAAATCCTCAGC 3'

Amplifications products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. A band at 300 base pairs (bp) represented *prkn*^{+/+} or *prkn*^{-/-} (ran separately; a sample with 300bp bands for both *prkn*^{+/+} and *prkn*^{-/-} is a *prkn*^{+/-} mouse). A band at 123bp represented *Sod2*^{+/+} and a band at 240bp represented *Sod2*^{-/-}.

Ambulatory activity (beam break)

To measure general locomotion and habituation, Micromax analyzer software and frames (Omnitech Electronics; Columbus, OH, USA) were used. Mice were single housed in new clean cages containing only a thin layer of bedding, food and water, and were loaded into the Micromax frames, following 1 hour of habituation in then dark. Testing was conducted for 4 hours. Infrared beam emitters and receptors were utilized to monitor the mice ambulatory activity, and beam breaks in 5 minute sampling bins were analyzed.

Nest Building

Nest building was qualitatively analysed as per Deacon et al., 2012⁴⁰⁹. Mice were single house in a new clean cage with with food, water and a single pressed cotton square ‘nestlet’. No habituation was needed. Mice were left in the new cage with the nestlet for 12 hours. Following the 12 hours, mice were removed from the testing cages and returned to their home cage. Videos were taken of the nests from the top and both sides and were blindly scored for quality on a scale of 1 to 5 based on the percentage of intact material remaining, cage floor area covered by the nesting material, and the height of the nest⁴⁰⁹.

Grip Strength

To measure forelimb grip strength independent of body weight, a grip strength meter (Columbus Instruments; Columbus, OH, USA) was used⁴²¹. Mice were held by the tail and lowered until they grab the grate of the meter with both forepaws. The mouse was then pulled horizontally gently but firmly until the mouse released the grate. The force reading in kilogram-force (kgF) was recorded. This was repeated until five successful pulls were recorded. Some examples of unsuccessful trials include: hindlimb contact with the grate, the mouse prematurely loosening its grip on the grate, or the body was not straight and horizontal to the table.

Rotarod

To monitor motor function, coordination and balance, the rotarod (IITC Life Sciences; Woodland Hills, CA, USA) was used as described previously^{422,423}. Mice were habituated to the rod by being placed on a rod rotating at 5rpm for 2 minutes, with the mice being replaced on the rod each time they fall off. Latency to fall, in seconds, was recorded after placing the mice on the rod accelerating from 4 rpm to 40 rpm for 5 minutes with a 10 minutes inter-trial time (ITI). The time was stopped when the mouse fell from the rod or when the mouse rotated around with the bar. Mice were tested for four trials per day for 3 days to assess learning.

Horizontal Ladder

To assess gait and coordination, we used the horizontal ladder, with irregularly spaced rungs, and measured the steps taken by an animal as it crossed it⁴⁰⁴. The horizontal ladder

is flanked by two plexiglass sidewalls. The ladder is 50 cm long and the rungs are spaced 0.5-2 cm apart. On day 1 (training day), each mouse was trained to walk across the ladder for 5 trials or until they walked across without stopping. On day 2 (testing day), the mice were filmed walking across the ladder for 4-5 trials. They mice were placed at the start end of the ladder and guided (if required), and the end of the ladder was their home cage to serve as an incentive to cross the ladder. The trials were videotaped and then analyzed, blindly, for correct stepping or errors (misses or cheats) for both forelimbs and hindlimbs.

DigiGait

To measure gait dynamics and posture we used the DigiGait™ imaging system (Mouse Specifics, Inc., Boston, MA, USA)⁴⁰⁵⁻⁴⁰⁷. Mice were first habituated to the room for 30 minutes before testing, then placed on a transparent treadmill equipped with an under-mounted camera, with the following conditions set; belt speed: 10-18cm/s, incline: 8 degrees, shutter speed: 22-24 for black mice. Approximately three seconds of video was recorded for each mouse. Built in algorithms analyzed the videos and quantified many gait and posture characteristics of which we chose to present stride length in centimeters (cm), stance width (cm), and step angle in degrees (°).

ROS (H₂O₂) measurements in tissues

Amplex® Red hydrogen peroxide/peroxidase assay kit (Invitrogen A22188) was used to monitor endogenous levels of H₂O₂ in mouse tissues. Pre-weighed hearts, cortex and midbrain pieces from mouse brains were homogenized on ice in the 1x reaction buffer provided, using a Dounce homogenizer (3 times volume to weight ratio). Homogenates

were diluted in the same 1x reaction buffer (10x and 5x). A serial dilution of the H₂O₂ standard provided was prepared (20, 10, 2 and 0 μM). 50 μL of standards and samples were plated in a 96 well black plate with clear flat bottom. The reaction was started by the addition of 50μL working solution which consisted of 1x reaction buffer, Amplex[®] red and horseradish peroxidase. The plate was incubated at room temperature for 30 minutes protected from light. A microplate reader was used to measure either fluorescence with excitation at 560 nm and emission at 590 nm, or absorbance at 560 nm. The obtained H₂O₂ levels (μM) were normalized to the tissue weight (g) or protein concentration (μg/μL).

Western blot and densitometry

Heart homogenates were run on 4-12 % Bis-Tris SDS-PAGE gels using MES running buffer. Proteins were transferred to PVDF membranes using transfer buffer, and immunoblotted for nitrotyrosine. Actin or Ponceau S staining were used as loading controls. For densitometry quantification, the signal intensity of protein nitrotyrosination from each sample was measured as pixel using Image J Software, and controlled for loading.

Superoxide (O₂⁻) measurements in tissue

To measure endogenous superoxide anion levels in mouse brains, we used high-performance liquid chromatography (HPLC), with the fluorescent probe dihydroethidium (DHE)⁴²⁴. Upon oxidation, DHE becomes positively charged and emits fluorescence. Two products emerge, ethidium from reaction with H₂O₂, and 2-hydroxyethidium

(2OHE) from the specific reaction with O_2^- . Using HPLC enables the separation and quantification of the two reaction products. Hemibrains pieces were cut, pre-weighed, and their weights equalized to 1-5mg. The pieces were incubated in 500 μ l PBS/diethylenetriaminepentaacetic acid (DPTA) containing 50 μ M DHE for 30 minute at 37°C. After incubation, the brains tissues were washed with PBS, dried, snap frozen in liquid nitrogen, homogenized and resuspended in 500 μ l acetonitrile, sonicated and centrifuged at 12,000g for 10 minutes at 4°C. The supernatants were transferred to a fresh tube and vacuum dried at low setting without heat. The pellets were resuspended in 120 μ l 20% methanol, 79.9% water and 0.1% trifluoroacetic acid, and a volume of 100 μ l was injected into the column. DHE was detected via UV (9.8-9.9 minutes), ethidium was detected via fluor (12.7-12.8 minutes) and 2OHE was detected via fluor (13.2 minutes).

Tissue processing, immunohistochemistry, and stereological cell counts

Mice were euthanized with a lethal injection of sodium pentobarbital. For immunohistochemistry, mice were transcardially perfused with 1x PBS followed by 4% paraformaldehyde (PFA). Brains were removed, post-fixed overnight in 4% PFA, and cryoprotected in 20% sucrose. Serial free-floating coronal sections were collected throughout the forebrain and midbrain. For estimates of dopaminergic neurons of the substantia nigra pars compacta (SNc), every 6th midbrain (Bregma -2.78 mm to -3.88mm Paxinos and Franklin, 2001) section (40 μ m) was collected and stained for tyrosine hydroxylase (TH-1) for stereological counts. All steps were done on a rotating shaker. Briefly, sections were washed in PBS, and endogenous peroxidase activity was quenched with 0.9% hydrogen peroxide in PBS. Sections were washed in PBS and blocked in 10%

normal serum in PBS to reduce non-specific signal. Sections were incubated overnight at 4°C in primary TH-1 antibody (1:10,000; Millipore, AB152) diluted in 1% normal serum in PBS-T. Sections were washed in PBS, and subsequently incubated for 2 hours in biotinylated rabbit secondary antibody (1:225) in 10% normal serum in PBS-T. Sections were then washed in PBS, and the signal was amplified with VECTASTAIN® Elite® ABC HRP Kit (Vector Labs, PK-6100), and visualized via standard DAB solution, 55mM DAB. Sections were washed with PBS, and mounted on charged slides, counterstained with Harris Modified Hematoxylin nuclei stain and dehydrated through a series of increasing ethanol concentration solutions and xylene, and coverslipped using Permount. A total of seven to eight 40um sections were examined within the rostral and caudal limits of the SNc. The number of dopaminergic neurons in the SNc were obtained via an unbiased stereological estimates with a 100x lens using Stereo Investigator (MicroBrightField). The total number of TH-1 positive neurons was determined by applying optical fractionation⁴²⁵.

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Differences between two groups were assessed using a Student t-test. Differences among 3 or more groups were assessed using a 1way or 2way ANOVA followed by Tukey, Dunnett or Bonferroni post hoc corrections (as indicated) to identify statistical significance. Subsequent post hoc tests are depicted graphically and show significance between treatments. For all statistical analysis

a cut-off for significance was set at 0.05. Data is displayed with p values represented as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

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Additional Information:

Data and materials availability: Original data associated with this study are available in the main text and extended data figures and tables; additional data will be made available upon request.

Supplementary Information is available for this manuscript.

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Chapter 5: General Discussion

5.1 Synopsis

Parkin-linked early-onset PD is a different disease entity when compared to idiopathic, sporadic or other monogenic forms of PD. Patient with EOPD-associated *PRKN* mutations develop the disease with an earlier age of onset, present with motor deficits in the absence of cognitive decline and LB pathology, and the disease appears localized to dopamine-producing neurons of the SNc (with LC nuclei affected in some cases) in the brainstem^{200,206-210}.

The *PRKN* gene was found to be linked to EOPD in 1998¹⁹³, and in the past two decades the field has mostly investigated parkin's E3 ubiquitin ligase function and its role in PINK1-mediated mitochondrial quality control (mitophagy)^{150,322}. Other functions of parkin have been identified, including in regulating mitochondrial antigen processing, suppression of tumour formation²⁹⁰, and pathways of inflammation signalling²⁹⁶⁻²⁹⁸. It has been widely viewed as a neuroprotective molecule³¹².

It has been convincingly shown that parkin has the aforementioned E3 ligase activity and associated functions. However, at the onset of our studies several critical elements in the biology of human parkin had led us to question whether these roles were indeed the 'essential' function(s) that are required for the maintenance of midbrain neuronal health. One, parkin's E3 ligase activity doesn't fully explain the exclusive degeneration of SNc DA neurons. An extensive list of parkin's putative substrates has been published, and in the absence of parkin no consistent accumulation of aberrant proteins across laboratories has been identified in different animal models and human brain. Additionally, within the

>100 EOPD-associated, mutant *PRKN* variants¹⁹⁵, several retain their E3 ligase activity²³³. Two, mitophagy pathways have been elegantly delineated in cellular paradigms; however, the overexpression of parkin and the high concentration of CCCP required to elicit a parkin recruitment response, put in question their physiological relevance. In addition, the concept of parkin-associated mitophagy has not yet been fully validated in neuronal cells and *in vivo*. For instance, aged, parkin-deficient mice do not display any abnormal striatal mitochondria when compared to WT littermates^{94,331}. Furthermore, parkin- and PINK1-independent mitophagy processes have been observed³²⁵⁻³²⁷. Three, and perhaps most important based on our findings, is the fact that many of the reported cell biological functions by parkin are dependent on the protein being soluble *in vitro* and *ex vivo*, which is distinct from its metabolism in human control brain.

Instead of E3 ligase activity as its essential function, we focused on additional clues in parkin biochemistry that we thought shed light on its essential function in DA neurons. One, preliminary findings that parkin shifts into an insoluble fraction in older human brains when compared to young cortices³³². Two, parkin is sensitive to redox reactions, inducing cysteine-mediated post-translational modifications, such as oxidation, S-nitrosylation and dopamine adduct formation, all of which alter its solubility and activity as an E3 ligase^{258,260,266,333}. Three, the primary structure of parkin reveals a high content of cysteine residues; these are known to carry redox-active thiol groups that are frequently involved in the maintenance of cellular integrity and organ health³³⁷⁻³⁴⁰. Four, several mouse models of parkin have been studied. Their authors concluded that these do

not lend themselves to robust modeling of PD³⁰¹, because they lack motor deficits and DA cell loss. However, they display alteration in the abundance and expression of several redox-sensitive proteins and enzymes (**Appendix V** – Supplementary tables)^{94,95}. In addition, in the absence of parkin, GSH has been observed to be dysregulated, but this finding had never been explained^{76,300}. All of these observations -when taken together- had suggested to us that parkin may play a role in redox biology.

Hence, we had hypothesized a cysteine-mediated, anti-oxidant function of parkin in DA-producing neurons that is independent but not exclusive of its E3 activity and related role in mitophagy. We posited that this redox function could explain the selective neuroprotection conferred by wild-type parkin to long-lived DA neurons in the SN.

5.2 Principal findings

Our collective work, comprising three manuscripts, resulted in several findings that established a novel role for parkin in redox physiology using both *in vitro*, *ex vivo* and *in vivo* paradigms. These included the examination of human tissues, genetic mouse models, several cell lines and highly purified recombinant proteins.

One, in > 60 human brains analyzed, parkin displayed progressive insolubility in the cortex and midbrain, with transition from a soluble to insoluble state occurring at a rate of ~90 per cent between the ages of 28 and 42 years. Interestingly, this coincides with the typical age of onset of parkin-linked EOPD. Additionally, we established a link between parkin's insolubility and its oxidative modifications of its thiol residues, which we began

to map to specific cysteine residues. Intriguingly, a similar age-dependent shift in parkin's solubility was absent in other human tissues, such as in skeletal muscle and spinal cord, and in aged, non-human species. This duality in functions, namely in E3 ubiquitin ligase activity and redox sensor, is not unprecedented, as the SAG protein displays both^{335,336}. Sustained oxidative stress has been widely interpreted by the field as detrimental to parkin because insolubility of the protein is followed by the loss of its E3 ligase activity. In contrast, we posit that parkin's age-associated oxidation and insolubility resulting from an increase in chronic oxidative stress reflect a crucial physiological role by which parkin maintains redox homeostasis *in vivo*. Moreover, E3 activity and redox biology are not entirely mutually exclusive. Low levels of oxidation has been shown to initially induce parkin-mediated ubiquitination *in vitro*^{256,258,260,357}

Our results do not exclude the possibility that other, non-oxidative PTMs of parkin, such as phosphorylation at Ser65 in humans^{237,271,426} and Ser77 in mice (identified in our work), sumoylation²⁶³, neddylation²⁶⁴, and truncation²⁶⁵, may also alter its solubility in human brain. However, we did not yet detect any of these PTMs by mass spectrometry for parkin isolated from human brains. We did however uncover a unique oxidation and dopamine-binding site at the primate-specific cysteine C95. This residue is located within the poorly studied linker region often omitted from structural studies due to its high flexibility. Recent reports have suggested that the linker region is crucial for the removal of parkin's autoinhibition and its activation^{319,427}. This is of importance, as PD is yet to be fully recapitulated in animal models, with the exception of select non-human primates

that have demonstrated the development of age-related dysfunction in the nigrostriatal system associated with an abnormal motor phenotype⁴²⁸.

Lastly, we validated parkin's progressive oxidation leading to its insolubility in cellular paradigms and in mouse brains under both mitochondrial and cytosolic stress conditions. Similarly, we have also begun to map parkin's biochemical sensitivity during an acute rise in pro-oxidants to select cysteine residues in mouse brains.

Two, we established a direct interaction between parkin and ROS, namely H₂O₂, *in vitro*. Parkin's ability to quench radicals in a concentration- and cysteine-dependent manner resulted in its direct oxidation and insolubility. Here we demonstrated that certain cysteine residues of parkin are more accessible for H₂O₂-dependent oxidation. We observed a sequential thiol oxidation starting with RING1, and followed by RING0, and RING2. At the highest H₂O₂ treatment concentration, almost all the cysteines were oxidized. At similar elevated H₂O₂ concentrations parkin's was observed to transition into the SDS-soluble fraction *in vitro*, in cellular paradigms and *ex vivo* in mouse brains.

Parkin's thiols are the mediators of this loss of solubility as they are exquisitely sensitive to oxidizing (H₂O₂), reducing (DTT) and electrophilic conditions (aminochrome, MPP+, rotenone, and CCCP). While rotenone and CCCP are known toxins and complex I inhibitors inducing mitochondrial deficits, they are also strong electrophiles, capable of reacting directly with thiol-rich proteins via their cysteine residues⁴²⁹⁻⁴³¹. We noted that some oxidation-induced insolubility is partially reversed by addition of thiol reducing

agent DTT and that aggregates formed adopt β -pleated sheet conformation akin to fibril formation in α -synuclein. Of note, most published reports on parkin's cellular function focus on soluble fractions or study the protein under reducing conditions⁴³², thereby completely missing conformations of parkin that are undetected and crucial for the understanding of parkin's essential role in the brain and its native metabolism.

Having a high content of cysteines residues with highly reactive thiol groups, allows parkin to directly interact with ROS, namely H_2O_2 . When compared to several other proteins, including PD-linked proteins (α -synuclein and DJ-1), BSA (containing, similarly to parkin, 35 cysteine residues), known cellular anti-oxidants (catalase and GSH) and other cysteine-rich E3 ligases (RNF43 and HOIP), parkin was much more efficient at reducing H_2O_2 to water, with the exception of catalase's enzymatic activity. Surprisingly, parkin was >5 times stronger than GSH at equimolar concentrations; GSH is the most abundant anti-oxidant in cells and tissues. We tested a wide range of concentrations for both parkin and H_2O_2 , to encompass human brain physiologically relevant measures, and used two different assays for validation. From our Western blot experiments we were able to crudely estimate the level of parkin expression in human brains, which was of about 1.5ng/mg of tissue. Based on our measures of H_2O_2 levels in human brains, we estimated the ratio of H_2O_2 (μM) per amount of parkin in tissues (ng) to be in the range of 200 to 8000 $\mu M/ng$. In investigating parkin's ability to directly interact and quench H_2O_2 *in vitro*, we used a wide range of H_2O_2 concentrations that, at the highest level, may seem physiologically non-relevant. However, we also consistently

used parkin concentrations higher than detected in human brain. Therefore, we were able to match closely the H₂O₂-to-parkin ratio detected in tissues, *in vitro*.

Three, consistent with its role as a redox sensor – in addition to directly interacting with H₂O₂, parkin also directly binds DA radicals. Our work builds on reports of DA-modified parkin²⁶⁰. Parkin's was able to bind dopamine radicals, such as highly reactive quinones, at distinct residues (C95, C166, C169, C182, C211, C238, C293, C360 and C365 – identified in additional work), we found evidence of its capacity to directly promote dopamine polymerization. Furthermore, in M17 cells we demonstrated a dose-dependent response in parkin's expression on cellular protection from exogenous dopamine exposure, which correlated with increased parkin-dopamine adduct formation, aggregation and insolubility, suggesting that parkin's biochemical changes, including HMW formation and insolubility, are non-toxic and potentially beneficial to maintain cellular health. Of interest, two PD-linked parkin mutants (G328E and C431F) had reduced ability to quench H₂O₂, and altered capacity to form aggregates, which ultimately affected their role in cell viability in response to dopamine stress.

In addition we detected parkin associated to neuromelanin in unperturbed human tissue samples. This is of likely relevance, because neuromelanin normally accumulates within healthy DA neurons and is lost as a result of nigral degeneration during parkin-linked ARPD and other forms of PD¹⁹⁹. Parkin's association with lysosomes, co-localizing with LAMP3 protein, suggests that both parkin and dopamine radicals that have polymerized within lysosomal structures are being sequestered in the form of neuromelanin. If validated, this would point at a role for parkin in promoting the sequestration of

dopamine, in order to avoid the toxic effects of its radicals; we noted with interest that parkin-null mouse models exhibit dopamine misprocessing, seen as increased midbrain DA levels, decreased endogenous DA release, and enhanced DA overflow^{217,299,300,396,397}. The ability of mice to better process dopamine metabolism, may explain the lack of a robust PD-like phenotype³⁰¹.

Four, supporting parkin's redox biochemistry are findings from human and mouse tissues. We observed positive correlation between age and H₂O₂ levels in human brains. Additionally, a greater increase was observed in ARPD parkin-deficient human brains, when compared to age-matched controls. In parkin-null mouse brains, H₂O₂ levels were not altered compared to WT animals; however, alterations in the expression of redox-sensitive proteins and enzymes have been reported^{94,95}. This could help explain the lack of a parkinsonian phenotype in these models. Furthermore, in a bi-genic mouse model, which was developed and characterized by our group, the *prkn*^{-/-} // *Sod2*^{+/-} animals combine a haploinsufficiency at the *sod2* locus with a loss of the *prkn* gene; there, we observed a rise in ROS levels in the *sod2*^{+/-} mouse, which was further increased by parkin deficiency. The rise in H₂O₂ levels in murine brain was complemented by an increase in chronic oxidative stress seen as the rise in irreversible markers of oxidation, namely protein carbonyls and nitrotyrosination. The effort to validate chronically elevated, oxidative stress in human ARPD brain is still underway.

Furthermore, in several cell lines the absence of parkin translated into a rise in H₂O₂ levels and an increase in cell death in response to pro-oxidant insults, including the

physiologically relevant rise in dopamine stress. Together, these findings led us to a fundamentally different interpretation of parkin's cysteine-based, highly variable oxidation: namely, that its expression is directly involved in the maintenance of redox homeostasis and brain health by lowering ROS, neutralizing RNS, and by sequestering RES, such as quinones (**Extended Data Figure 2.9**).

Five, we uncovered a reciprocal relationship between parkin expression and GSH metabolism *in vivo* in both human and mouse brains. As mentioned earlier, the exquisitely sensitive thiol group of cysteine residues undergo a multitude of redox reaction ultimately affecting the maintenance of overall redox balance and cellular and organ health^{339,340}. In parkin's absence, we observed elevated levels of GSH; decreased levels of GSSG; increased levels of GSH:GSSG; and unchanged levels of total glutathione (GSH+GSSG). We postulated that parkin's presence reduced cellular needs for higher levels of free GSH. Surprisingly, *de novo* synthesis of GSH did not explain the altered metabolism downstream of parkin deficiency, as *GCLM* or *GCLC* mRNA levels were unaltered. However, using the Tietze-method to measure GSH levels in murine brain, we observed an increase in GSH and reciprocal decrease in GSSG levels in *prkn*^{-/-} mice, akin to what was observed by Itier et al. in 2003³⁰⁰, which we attributed to an increase in GR enzymatic activity, known to recycle GSSG back to GSH^{382,383}. This still requires further testing in dynamic studies in cell and murine models.

Under stress condition, Nrf2-is known to upregulate the expression of several cellular defense genes. In addition it has been reported that it can also mediate the regulation of

GSH redox status by GR-mediated recycling. This pathway is critical for cell survival during oxidative stress, and is independent of the induction of GSH biosynthetic enzymes⁴³³. The Nrf2-mediated pathway could explain the reports of parkin's perinuclear localization^{265,434}. However, the delineation of that mechanism has yet to be elucidated. Nevertheless, we found that parkin itself contributed to increased GSH levels by direct thiol-mediated recycling of GSSG, resulting in one of parkin's cysteines S-glutathionylated and one molecule of GSH. Parkin's reversible S-glutathionylation further solidifies the versatile reactivity of its thiol groups and highlights the relevance of parkin's integration into redox chemistry. Of note, yet again parkin's primate-specific residue C95, which is located within -what is now referred to as- the 'activating domain of parkin'²⁵⁴, is the target of another post-translational modification, in this case S-glutathionylation (**Figure 3.4f**). Importantly, the significantly altered GSH metabolism in parkin's absence, and the effective compensation by higher amounts of available (reduced) GSH, could explain the lack of neuronal death and related parkinsonian phenotype in virtually all genomically deficient *prkn*-null mice.

Six, we generated and characterized a murine model for chronic, parkin-linked oxidative stress, which combined haploinsufficiency at the *sod2* locus with parkin deficiency. Age-dependent analysis revealed that parkin protected against a sustained increase in excessive oxidation resulting in irreversible markers of oxidative stress. Nevertheless, the increase in oxidative stress did not result in a parkinsonian phenotype, such as shown through motor deficits or a decline in DA cell numbers. Our observed increase in GSH levels in the bi-genic mouse, along with reports of enhanced VDAC-mediated shuttling

of superoxide into the cytosol and its subsequent reduction by cytosolic sod1 in response to a decrease in mitochondrial sod2 activity³⁷⁸, most likely underlies the lack of a more severe phenotype. Hence, our bi-genic modeling did not yet overpower the compensatory changes that seem to be present throughout adulthood in mice.

5.3 Future Directions

Taken together, our results demonstrated a strong link between parkin's thiol-mediated biochemistry and downstream cellular events. We demonstrated that native parkin resides in multiple oxidation states *in vivo*, both reversible thiol-mediated higher molecule weight species, and irreversibly oxidized, fully-aggregated and insoluble forms. In addition, its substantial contribution to the cellular thiol pool reciprocally affected GSH's metabolism in human brain. However, more work in the future is needed to solidify parkin's contribution to redox pathways and to convincingly delineate its neuroprotective effects in DA neurons.

A more extensive and precise mapping of oxidative modifications of parkin that occur in normal human brain, including those induced by ROS, RNS and RES attacks, is needed. Fine mapping is ongoing in the field, and several structural studies have shown the importance of key residues in the activation of the protein. The finding that some of these residues may be oxidized in human brain will inform those studies. In addition, a larger-scale assessment of the diversity and quantity of oxidative modifications in mouse brains parkin by LC-MS/MS, for example in our MPTP-exposed *vs.* untreated mouse model, in order to map additional *bona fide* oxidation sites that occur *in vivo*, is required. We have

also not yet quantitatively assessed cysteine-mediated oxidative modifications between soluble and insoluble fractions in human brains, from young and old human brain extracts, respectively. This could help shed light on select cysteine residues involved in the oxidation-dependent aggregation and insolubility of parkin.

The heterogeneous nature of cysteine oxidations, likely responsible for the lower yield observed by IP and LC-MS/MS coverage in human and mouse brains, presents a challenge. Additionally, due to the transient nature of S-glutathionylation (as well as of S-nitrosylation²⁵⁸) and our LC-MS/MS analysis performed under reducing conditions, we have not yet validated parkin's S-glutathionylation and S-nitrosylation *in vivo*. Future experiments will require further optimization of our workflow taking into consideration the sensitivity of thiol groups to both reducing and oxidizing conditions, a greater yield of enriched parkin, and thus, significantly larger amounts of human tissues, including from the *S. nigra* and striatum. In addition, access to human midbrain tissues and related *S. nigra* organoids^{358,435-437}, generated from parkin-deficient EOPD patients, or patient with parkin point mutations, will provide clarification as to which structural domains of parkin are essential to its redox activity, more sensitive to oxidation resulting in aggregation and insolubility, and more able to promote dopamine polymerization and associate with neuromelanin.

Although *PRKN* mRNA is continuously transcribed in human *S. nigra* neurons with age, when parkin protein signal had already shifted into an insoluble fraction, the rate of translation for *PRKN* transcripts into protein under redox stress conditions, such as in

aged human DA neurons, remains to be studied. In addition further validation of carbonylation and nitrotyrosination, as well as human brain-specific changes in redox state-related transcriptomes in *PRKN*-genotyped, age-matched, human brain tissue specimens is needed. Lastly, the characterization of additional glutathione-regulating enzyme levels *in vivo* in order to validate our model that GR activity is significantly increased in EOPD brains is required.

In addition to biochemically mapping parkin's oxidative modifications, the ability to detect and observe the different species of parkin in human and mouse brains has been a challenge in the field, due to the lack of specific antibodies. We have developed and are currently further characterizing (in a fourth manuscript) novel parkin antibodies designed to detect reduced and oxidized species. These antibodies allowed us for the first time to consistently detect parkin in human brain by Western blotting, IHC and IIF, and will be useful in the future to better visualize parkin's localization in human brains, such as by electron microscopy. In addition, using our new antibodies will hopefully allow us in future experiments to precisely quantify by ELISA the exact amount of parkin in human midbrains, in order to better elucidate its neuroprotective function in a physiologically relevant manner.

A better understanding of parkin's biochemistry will help us to develop and characterize better animal models of early-onset parkinsonism. For instance, the inhibition of cytosolic GSH pathway, through the disruption of the mouse glutamate-cysteine ligase modifier subunit (*Gclm*) gene, coding for the rate-limiting enzyme in the GSH-

biosynthesis pathways⁴¹², or treatment with BSO⁴¹³, in addition to parkin deficiency, may reveal a parkin-dependent neuroprotective function, and result in a parkinsonian phenotype. Additionally, neuromelanin, sequestering oxidized dopamine, deposits are specific to humans and primates, and are not observed in aging mouse models, potentially explaining the lack of PD pathogenesis. Hence, the addition of dopamine toxicity through L-dopa administration or monoamine oxidase-B (MAO-B) inhibition^{414,415} to our mouse models may result in the so desired PD phenotype. Furthermore, experiments exploring neuromelanin production in mice, using tyrosinase overexpression, to produce features of parkinsonism may be a useful tool³⁴⁶.

Our observation that parkin's diverse activities, including the sensing of oxidative stress, the physical response to it, and the co-regulation of anti-oxidant outcomes, are anchored in traditional redox chemistry provides a biochemical and unifying explanation for its role in neuroprotection, and may finally answer the question of what is the essential function of parkin in human midbrains conferring its selective neuroprotection to DA neurons. In addition, where the field is mostly focusing on targeting parkin's E3 ligase activity, through small molecule activators of the protein, as a therapy; the concept of parkin as a redox molecule opens the door to exploring better cause-directed therapies, in addition a better stratification of patients. Revisiting therapeutic strategies in EOPD, such as via viral vector delivery of *PRKN* cDNA constructs^{8,438} and anti-oxidant therapy^{359,419,420} may prove to be useful.

Appendices

Appendix I – References

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Appendix II – PD animal models.

Rattus norvegicus (Rat)

Types	Promoter	SN neuron loss	Inclusion bodies	Motor impairment
HUMAN α-SYNUCLEIN				
A30P	BA	+ (DA loss)	+	ND
WT	CBA	+	+	+
A53T	CBA	+	+	+
WT/A30P/A53T	PGK	+ (DA loss)	+	ND
WT	CMV	+ (DA loss)	–	ND
WT, S129D, S129A	CMV	+ (DA loss)	+	ND
A53T	CBA	+ (DA loss)	+	ND
WT, S87A	ND	+ (DA loss)	+	+
S87E	ND	–	+	–
WT	SYN 1	+ (DA loss)	+	+
HUMAN LRRK2				
WT	SYN 1	–	–	ND
G2019S	SYN 1	+	–	ND
DJ-1 RAT HOMOLOG				
WT (KO -Exon 5)	–	+ (DA loss)	–	+
PINK-1 RAT HOMOLOG				
WT (KO -Exon 4)	–	+ (DA loss)	–	+

Drosophila melanogaster (Fruit fly)

Types	Driver	DA neuron loss	Inclusion bodies	Motor impairment
Human α-synuclein				
WT/A30P/A53T	elav-GAL 4	+	+	+
WT/A30P/A53T	elav-GAL 4	+	+	ND
WT	elav-GAL 4	–	+	+
S129D	elav-GAL 4	+	+	ND
S129A	elav-GAL 4	–	+	ND
WT 71–82 removed	elav-GAL 4	–	–	ND
WT 1–120 trunc.	elav-GAL 4	+	+	ND
WT 1–78 trunc.	elav-GAL 4	+	+	ND
Human LRRK2				
WT	elav-GAL 4	+	ND	+
G2019S	elav-GAL 4	+	ND	+
I2020T	elav-GAL 4	–	–	+
WT	ddc-GAL4	–	–	–
G2019S	ddc-GAL4	+	–	+
Y1699C	ddc-GAL4	+	–	+
G2385R	ddc-GAL4	+	–	–
DJ-1 FLY HOMOLOG				
DJ-1 β part deletion	–	–	–	+
DJ-1 α null	–	–	–	ND
DJ-1 β null	–	–	–	ND
DJ-1 β null	–	–	ND	ND
DJ-1 α RNAi	–	+	ND	–
DJ-1 β null	–	–	ND	–
PINK-1 FLY HOMOLOG				
Kinase domain	–	–	ND	+
UTR + part of exon 1	–	+	ND	+

Appendix II: Animal models of PD.

Summary table of rat and fly models of autosomal dominant (α -synuclein and LRRK2), and autosomal recessive (PINK1 and DJ-1) PD. WT, wild-type; BA, β -actin; CBA, chicken β -actin; PGK, phosphoglycerate kinase; CMV, cytomegalovirus; SYN 1, synapsin 1; elav, embryonic lethal – abnormal vision; ddc, dopa decarboxylase; RNAI, RNA interference; UTR, untranslated region; and ND, no data reported.

Appendix III – PD-linked parkin point mutations.

	Mutation	Domain	Predicted structural rationale	Biochemical effect
Zinc-coordinating residues	C166Y	UPD	Coordinates UPD Zn2	Reduced solubility (Wang <i>et al</i> , 2005)
	C212G	UPD	Coordinates UPD Zn1	
	C212Y			
	H215Q	UPD	Coordinates UPD Zn1	
	C238Q	RING1	Coordinates RING1 Zn1	- Reduced complex formation (Van Humbeeck <i>et al</i> , 2008) - Reduced solubility (Wang <i>et al</i> , 2005) - Autoubiquitination, but reduced substrate ubiquitination (Sriram <i>et al</i> , 2005)
	C253Y	RING1	Coordinates RING1 Zn2	
	C253W			
	R256C	RING1	Creates a competing Cys for RING1 Zn2 binding	
	H257R	RING1	Coordinates RING1 Zn2	- Reduced solubility (Wang <i>et al</i> , 2005; Gu <i>et al</i> , 2003)
	C289G	RING1	Coordinates RING1 Zn2	
	R334C	IBR	Creates a competing Cys for IBR Zn1 binding	- Reduced solubility (Wang <i>et al</i> , 2005)
	R402C	Linker helix	May interfere with Zn1 binding of RING1	- Reduced solubility (Gu <i>et al</i> , 2003)
	R402H			
	C418R	RING2	Coordinates RING2 Zn1	- Reduced solubility (Wang <i>et al</i> , 2005; Hampe, 2006)
	C441R	RING2	Coordinates RING2 Zn1	
Domain residues	P153R	UPD	In UPD Zn1 binding loop	- Ligase dead (Sriram <i>et al</i> , 2005) - Reduced mitochondrial localization (Matsuda <i>et al</i> , 2010)
	T173M	UPD	In UPD Zn2 binding loop	
	T240R	RING1	In RING1 Zn1 binding loop	
	T240M		May affect E2 binding	
	V258M	RING1	RING1 core residue, may affect linker helix binding	- Reduced solubility (Wang <i>et al</i> , 2005) - Increased ubiquitination activity (Sriram <i>et al</i> , 2005) - No mitophagy (Narendra <i>et al</i> , 2010; Geisler <i>et al</i> , 2010)
	Y267H	RING1 helical	Core residue of RING1 helical domain	
	C268R	RING1 (helical)	Core residue of RING1 helical domain	
	R275N	RING1 (helical)	RING1 helical domain	
	R275W		core residue	
	R275T			
	I298S	RING1	RING1 core residue	
	I298L			

Domain residues	T351P	IBR	Disrupt IBR β -sheet, next to Zn-coordinating Cys	IBR domain destabilisation (Beasley <i>et al</i> , 2007)
	R366T R366Q	IBR	IBR core residue, also on interface with RING1	
	P437L P437I	RING2	Near Zn1 binding site	Ligase dead (Sriram <i>et al</i> , 2005)
Residues involved in the ubiquitination mechanism	D243N	RING1	Forms contact with conserved Lys on E2 helix 1	
	C431F	RING2	Active site Cys	- Reduced solubility (Wang <i>et al</i> , 2005; Sriram <i>et al</i> , 2005) - Increases autoubiquitination (Sriram <i>et al</i> , 2005) - No substrate ubiquitination (Sriram <i>et al</i> , 2005) - Reduced translocation to mitochondria (Lazarou <i>et al</i> , 2013)
	E444Q	RING2	Potential catalytic triad residue (unconfirmed)	
Residues at the interface between the UPD/RING0 and RING2 domains	S145N	UPD	Destabilises interface?	
	M192L	UPD	Destabilises interface?	
	M192V	UPD	Destabilises interface?	
Residues at the interface between the RING1 and the linker region	S193I	UPD	Destabilises interface?	
	G429D	RING2	Destabilises interface?	
	G430D	RING2	Destabilises interface?	- Ligase dead (Chew <i>et al</i> , 2011) - Increased auto-ubiquitination (Sriram <i>et al</i> , 2005) - Reduced substrate ubiquitination (Sriram <i>et al</i> , 2005)
Residues at the interface between the RING1 and the linker region	M458L	RING2	Near RING2 UPD interface but exposed	
	A230T	RING1	May create RING1/RING2 interface. May affect Linker helix binding. May affect RING1 structure	
Residues at the interface between the RING1 and the linker region	R234Q	RING1	May affect linker helix binding	
	A398T	Linker helix	Disrupts linker helix binding	
	A401D	Linker helix	Disrupts linker helix binding	

Residues at the interface between the RING1 and the IBR domains	N273S	RING1	Destabilises interface?	
	E310D	RING1	Destabilises interface?	
	Q311H	RING1	Destabilises interface?	
	G328E	RING1	Loop between RING1 and IBR	- Increased auto-ubiquitination (Sriram <i>et al</i> , 2005)
			Destabilises interface?	- Reduced substrate ubiquitination (Sriram <i>et al</i> , 2005) - Reduced solubility (Wang <i>et al</i> , 2005)

Residues with unknown effects on parkin's function	H200Q	UPD	Exposed in UPD	
	V244I	RING1	On RING1, exposed	
	H265R	RING1	On RING1, exposed	
		helical		
	D280N	RING1	β-sheet insertion in RING1	Reduced solubility (Wang <i>et al</i> , 2005)
	L283P	RING1	β-sheet insertion in RING1	
	G284R	RING1	β-sheet insertion in RING1	
	T415N	RING2	Exposed in RING2.	Ligase dead (Sriram <i>et al</i> , 2005; Matsuda <i>et al</i> , 2010)

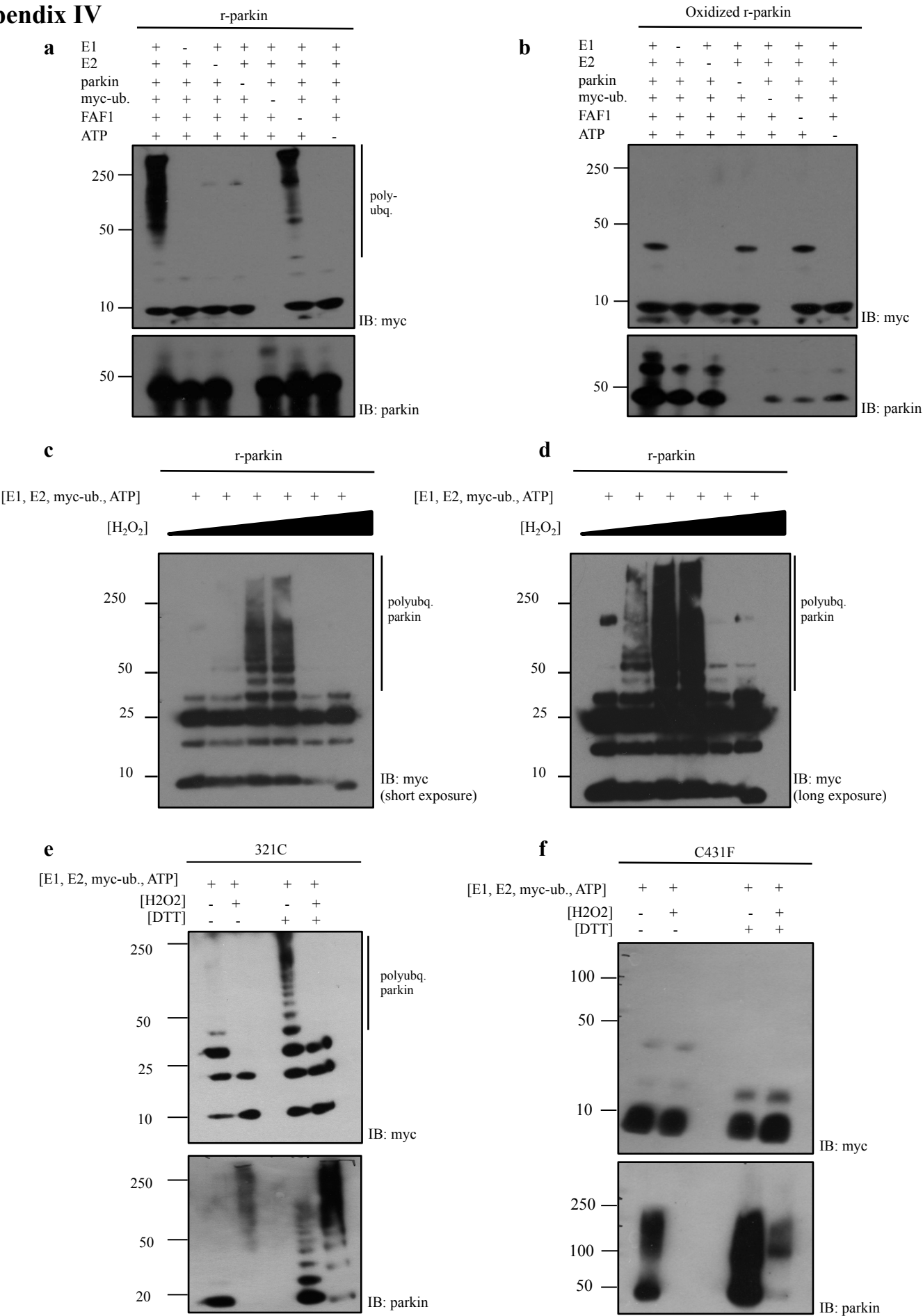
Appendix III: EOPD-linked parkin mutations.

Summary table of select known parkin mutations associated with EOPD. The domain localization of the mutations, and their structural and biochemical effect are displayed.

UPD, unique parkin domain renamed RING0 and discovered by Hristova *et al.*, 2009.

Modified from Wauer *et al.*, 2013.

Appendix IV



Appendix IV: Additional data – parkin-mediated ubiquitination.

Parkin's E1, E2, ubiquitin and ATP-dependent E3 ligase activity was assessed under **a**, control conditions; and **b**, post oxidation with H₂O₂ for autoubiquitination and the ubiquitination of a known substrate (FAF1). The ubiquitination activity was monitored by immunoblotting for myc to visualize myc-ubiquitin chains. **c-d**, Parkin's ligase activity was monitored under increasing concentrations of H₂O₂ (2μM to 20mM). The ubiquitin ligase activity of **e**, a N-terminally truncated parkin (321C); and **f**, a PD-linked E3-dead parkin mutant (C431F) were monitored under oxidising and/or reducing conditions.

Appendix V – Supplementary tables.

a

Name	Protein/Enzyme	Redox Sensitivity
Pyruvate dehydrogenase E1	Enzyme	Yes
NADH-ubiquinone oxidoreductase 24-kDa subunit	Enzyme	Yes
NADH-ubiquinone oxidoreductase 30-kDa subunit		
Cytochrome c oxidase, subunit Vb	Enzyme	Yes
Peroxiredoxin 2	Enzyme	Yes
Peroxiredoxin 6		
Peroxiredoxin 1		
Lactoylglutathione lyase (glyoxalase I)	Enzyme	Yes
Profilin II	Protein	Yes
Hypothetical Protein dJ37E16.5- similar to nitrophenylphosphatases	Enzyme	Yes
Vacuolar protein sorting-29	Protein	No
α -Crystallin, chain B	Protein	Yes
Heterogeneous nuclear ribonucleoprotein A1	Protein	Yes
Lasp-1	Protein	No
Ratio of redox sensitive:not redox sensitive	12:2	

b	Name	Protein/Enzyme	Redox Sensitivity
	Fructose-bisphosphate aldolase C	Enzyme	Yes
	ATP synthase α chain, mitochondrial	Enzyme	Yes
	Citrate synthase	Enzyme	No
	Dihydrolipoamide dehydrogenase (E3)	Enzyme	Yes
	Succinate dehydrogenase flavoprotein subunit	Enzyme	Yes
	Glyceraldehyde-3-phosphate dehydrogenase	Enzyme	Yes
	GTP:AMP phosphotransferase mitochondrial	Enzyme	No
	Pyruvate kinase, M2 isozyme	Enzyme	Yes
	L-Lactate dehydrogenase A chain	Enzyme	Yes
	Malate dehydrogenase, cytoplasmic	Enzyme	Yes
	NADH-ubiquinone oxidoreductase 51-kDa subunit (complex I)	Enzyme	Yes
	Pyruvate dehydrogenase E1 component β subunit	Enzyme	Yes
	Phosphoglycerate kinase 1	Enzyme	Yes
	Succinyl-CoA ligase β -chain, mitochondrial	Enzyme	Yes
	Acetyl-CoA acetyltransferase, mitochondrial (thiolase)	Enzyme	Yes
	Ubiquinol-cytochrome c reductase complex core protein I (complex III)	Enzyme	Yes
	β -Adrenergic receptor kinase 1	Enzyme	No
	Proto-oncogene C-crk (adapter molecule crk, member of adapter protein family)	Protein	Yes
	Dihydropyrimidinase-related protein-2 (CRMP-2)	Protein	Yes
	Ser/Thr protein phosphatase 2B catalytic subunit, α isoform (PP2B)	Enzyme	Yes
	Ser/Thr protein phosphatase PP1- β catalytic subunit	Enzyme	Yes
	Protein tyrosine phosphatase, non receptor type 1-1	Enzyme	Yes
	14-3-3 prot	Protein	No
	MAGUK p55 subfamily member 3	Protein	No
	rho GDP-dissociation inhibitor 1	Protein	No
	N-ethylmaleimide-sensitive fusion protein (NSF)	Enzyme	Yes
	Syntaxin 1B	Protein	Yes
	Syntaxin-binding protein 1	Protein	Yes
	Septin 5	Protein	Yes, contain cys residues that form disulfide bonds with each other
	Septin 7		
	Septin-like protein KIAA0202 (Septin 8)		
	Hypothetical protein FLJ10849 (Septin 2 or 6 homologue)		
	Stress-70 protein (mortalin)	Protein	Yes
	Heat-shock cognate 71-kDa protein	Protein	Yes
	Carbonyl reductase [NADPH] 1	Enzyme	Yes
	Glutathione S-transferase P 2	Enzyme	Yes
	Glyoxalase I	Enzyme	Yes
	Thioredoxin reductase	Enzyme	Yes
	ARP2/3 complex 20-kDa subunit	Protein	No

Adenyl cyclase-associated protein 1	Protein	No
Dynamin-1	Protein	Yes
Tubulin α 1 chain	Protein	Yes
Proteasome subunit beta type 5	Protein	Yes
Ubiquitin carboxyterminal hydrolase L1	Enzyme	Yes
Deubiquitinating enzyme (Ubiquitin thioesterase) OTUB1	Enzyme	Yes
Acyl-CoA dehydrogenase, very-long chain specific	Enzyme	Yes
Propionyl CoA carboxylase α chain	Enzyme	Yes
Lysophospholipase 1 (phospholipase B)	Enzyme	Yes
Tyrosyl tRNA synthetase	Enzyme	Yes
Threonyl tRNA synthetase	Enzyme	Yes
Glutamine synthetase	Enzyme	Yes
Serine racemase	Enzyme	Yes
Probable ATP-dependent RNA helicase p47	Enzyme	No
Poly(rC)binding protein	Protein	No
4-Aminobutyrate aminotransferase, mitochondrial (GABA transaminase)	Enzyme	Yes
Tyrosine 3-hydroxylase	Enzyme	Yes
Serum albumin	Protein	Yes
ADP-ribosylarginine hydrolase	Enzyme	No
Carbonic anhydrase II	Enzyme	Yes
Low-affinity immunoglobulin epsilon FC receptor	Protein	No
Ribose-phosphate pyrophosphokinase I	Enzyme	No
Voltage-dependent anion-selective channel protein 1	Protein	Yes
Spermidine synthase	Enzyme	No
γ Enolase	Enzyme	Yes
NADH-ubiquinone oxidoreductase PDSW subunit HS homolog	Enzyme	Yes
MAGUK p55 subfamily member 6	Protein	Yes
Sumo-1 activating enzyme subunit 2 (Ubiquitin-like 1-activating enzyme)	Enzyme	Yes
MAGUK p55 subfamily member 2	Protein	No
Sorting nexin 5	Protein	No
78-kDa glucose-regulated protein (binding immunoglobulin protein; heat shock 70 kDa protein 5)	Protein	Yes, "protect cells against oxidative stress"; it is overexpressed in conditions of oxidative stress
Heat-shock-related 70 kDa protein 2		
Heat-shock 70-related protein APG-1		
T-complex protein 1, α subunit B	Protein	Yes
Aconitate hydratase, mitochondrial (aconitase)	Enzyme	Yes
γ -Actin	Protein	Yes
Spectrin α chain, brain	Protein	Yes
Ubiquitin-activating enzyme E1 type 1	Enzyme	Yes
Acyl-coenzyme A oxidase 1, peroxisomal	Enzyme	No
Acyl CoA thioester hydrolase	Enzyme	Yes
Sodium/potassium-transporting ATPase α -3 chain	Enzyme	Yes
Calretinin	Protein	Yes
ATP-dependent RNA helicase DDX19, dead box protein	Protein	No
Ratio of redox sensitive: not redox sensitive	64:18	

Appendix V: Alterations in protein and enzyme expression levels in *prkn*^{-/-} mice.

Summary table of changes in abundance and expression levels of proteins and enzymes in *prkn*^{-/-} mice from unbiased proteomic analyses published by **a**, Palacino et al., 2004, and **b**, Periquet et al., 2005. The combined work identified dysregulation in 92 proteins downstream of loss of parkin, of which 55 were enzymes and 72 were redox-sensitive.

Appendix V – Additional scientific contribution.

In collaboration with Dr. Bojan Shutinoski and the Schlossmacher team, working on identifying a role for LRRK2 in immune defenses through modulation of inflammatory responses during microbial infection, I measured endogenous ROS levels, namely H_2O_2 , in mouse brains and spleens. When compared to mock infected animals, mice with the PD-associated G2019S LRRK2 variant, with hyper kinase activity, showed increased levels of H_2O_2 in their brains and spleens post *Salmonella typhimurium* (ST) infection. Of interest, ST infection does not cross the blood brain barrier, therefore this work is important in elucidating a systemic role for LRRK2 in the periphery, ultimately affecting brain health.

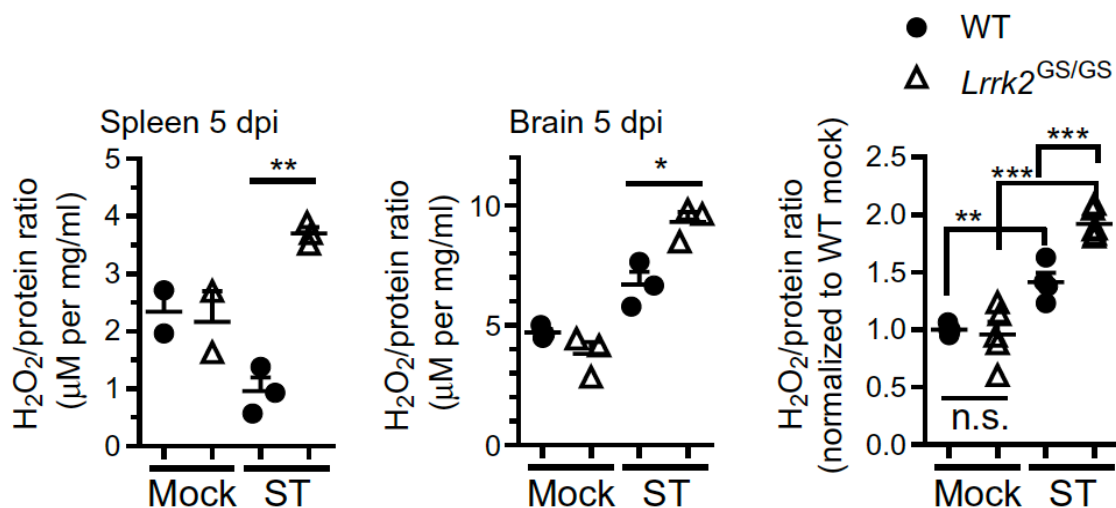


Figure taken from Bojan Shutinoski*, Mansoureh Hakimi1*, Irene E. Harmsen, Michaela Lunn, Juliana Rocha, Nathalie Lengacher, Yi Yuan Zhou, Jasmine Khan, Angela Nguyen, Quinton Hake-Volling, **Daniel El-Kodsi**, Juan Li, Azadeh Alikashani, Claudine Beauchamp, Jay Majithia, Kevin Coombs, Derya Shimshek, Paul C. Marcogliese, David S. Park, John D. Rioux, Dana J. Philpott, John M. Woulfe, Shawn Hayley, Subash Sad, Julianna J. Tomlinson, Earl G. Brown, Michael G. Schlossmacher. **Lrrk2 alleles modulate inflammation during microbial infection of mice in a sex-dependent manner.** *Sci Transl Med*, 2019; 11 (511). doi:10.1126/scitranslmed.aas9292