

**Genome-Wide Screen Identifies Novel Genes Involved
in Mitochondrial Quality Control**

By Andy Cheuk-Him Ng

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Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine,
University of Ottawa, Ontario, Canada

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Abstract

In addition to ATP generation, mitochondria are essential in various cellular processes ranging from biosynthetic pathways, apoptosis, cell cycle progression, and calcium buffering. Studies in living cells have now firmly established that mitochondria exist as a dynamic network sculpted by fission and fusion reactions, rather than separated, individual organelles. Not surprisingly, mutations in genes involved in mitochondrial dynamics and quality control lead to human diseases such as Charcot-Marie-Tooth disease type 2A, Optic atrophy, and autosomal recessive Parkinson disease. I have designed a high-throughput protocol to permit genome-wide screening for novel genes that are required for normal mitochondrial morphology. I have executed a genome-wide RNA interference screen and identified several novel genes required for mitochondrial dynamics in addition to known regulators of mitochondrial dynamics. A detailed high-throughput genome-wide screening protocol is presented. I have shown that *TID1*, a gene identified from the screen, has a dual-role in maintaining the integrity of mitochondrial DNA and preventing the aggregation of complex I subunits. My analysis of the mitochondrial role of TID1 supports the existence of a TID1-mediated stress response to ATP synthase inhibition. The genome screen also identified the novel gene *ROMO1* as essential for normal mitochondrial morphology. I have shown that ROMO1 may have an additional role in maintaining mitochondrial spare respiratory capacity, possibly by affecting cellular substrate availability. Finally, in a collaborative effort, we have shown that homozygous mutations in the mitochondrial fusion gene *MFN2* lead to multiple symmetric lipomatosis (MSL) associated with neuropathy. Mechanistically, this mutation reduces MFN2 homocomplex formation. Taken together, these results show the utility of genome-wide screening in identifying genes involved in mitochondrial quality control.

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List of Abbreviations

3-NP	3-nitropropionic acid
AAV	Adeno-associated virus
Acetyl-CoA	Acetyl coenzyme A
ATP	Adenosine triphosphate
ATP5A	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
ATPIF1 (IF1)	ATPase inhibitory factor 1
BMH	Bismaleimidohehexane
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BTICs	Breast tumor initiating cells
cAMP	Cyclic adenosine monophosphate
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
cDNA	Complementary deoxyribonucleic acid
CK	Creatine kinase
CMT2A	Charcot-Marie-Tooth disease type 2A
Co-IP	Co-Immunoprecipitation
CON	Control
CREB/CREB1	cAMP responsive element binding protein 1
DAPI	2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride
DCF-DA	2',7'-Dichlorofluorescein diacetate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRP1	Dynamin 1-like
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
eGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-bis(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid
EMF (Δp)	Electromotive force
EMG	Electromyogram
ER-mito	Endoplasmic reticulum-mitochondria
ETC	Electron transport chain
EtOH	Ethanol
FADH ₂	Flavin adenine dinucleotide (hydroquinone form)
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FCS	Fetal calf serum
FFA	Free fatty acid
FILA	Fatal infantile lactic acidosis
FIS1	Fission 1 (mitochondrial outer membrane) homolog
Fzo	Fuzzy onions
GED	GTPase effector domain
GFP	Green fluorescent protein

HbA1C	Hemoglobin A1c
HDL	High-density lipoprotein
HMSN	Hereditary motor sensory neuropathies
HR1	Heptad repeat 1
HR2	Heptad repeat 2
HSP40	Heat shock protein 40 kDa
HSPA9/mtHSP70	Heat shock 70kDa protein 9 (mortalin)
IgG	Immunoglobulin G
LHON	Leber hereditary optic neuropathy
Mdj1p	Mitochondrial DnaJ homolog
MERRF	Myoclonic epilepsy with ragged-red fibers
MFF	Mitochondrial fission factor
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MiD49	Mitochondrial dynamics protein of 49 kDa
MiD51	Mitochondrial dynamics protein of 51 kDa
MOPS	3-(N-Morpholino)propanesulfonic acid
MPC	Mitochondrial pyruvate carrier
MSL	Multiple symmetric lipomatosis
MT-CO1	Mitochondrially encoded cytochrome c oxidase I
MT-CO2	Mitochondrially encoded cytochrome c oxidase II
MT-RNR1	Mitochondrially encoded 12S RNA
mtDNA	Mitochondrial DNA
MTR	MitoTracker Red CM-H ₂ Xros
MTX2	Metaxin 2
NAC	N-Acetylcysteine
NADH	Nicotinamide adenine dinucleotide
NCV	Nerve conduction velocity
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)
Neg control	Negative control
NP-40	Nonyl phenoxypolyethoxylethanol
OCR	Oxygen consumption rate
OMIM	Online Mendelian Inheritance in Man
OPA1	Optic atrophy 1
OPA1-L	Optic atrophy 1-long isoform
OPA1-S	Optic atrophy 1-short isoform
Park2/Parkin	Parkin RBR E3 ubiquitin protein ligase
PBS	Phosphate buffered saline
PD	Parkinson Disease
PINK1/PARK6	PTEN induced putative kinase 1
PKA	Protein kinase A
Pos control	Positive control
PRDX	Peroxiredoxin 1
QPCR	Quantitative polymerase chain reaction
RHOT1/2	Ras homolog family member T1/Ras homolog family member T2

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROI	Region of interest
ROMO1/MTGM	Reactive oxygen species modulator 1/Mitochondrial targeting GxxxG motif
ROS	Reactive oxygen species
rPFO/XF PMP	Recombinant perfringolysin/XF plasma membrane permeabilizer
rRNA	Ribosomal ribonucleic acid
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SRC	Spare respiratory capacity
TC	Tissue culture
TCA cycle	Tricarboxylic acid cycle
TFAM	Transcription factor A, mitochondrial
TID-L	Tumorous imaginal discs 1-long isoform
TID-S	Tumorous imaginal discs 1-short isoform
TID1/DNAJA3	Tumorous imaginal discs 1/DnaJ (Hsp40) Homolog Subfamily A Member 3
TMPD	<i>N,N,N',N'</i> -Tetramethyl- <i>p</i> -phenylenediamine
TMRE	Tetramethylrhodamine ethyl ester
TOMM20	Translocase of outer mitochondrial membrane 20 homolog
TORCs/CRTC	Transducer of regulated CREB protein 2/CREB regulated transcription coactivator 2
tRNA	Transfer ribonucleic acid
U2OS	Human osteosarcoma cell line
UCP1	Uncoupling protein 1 (mitochondrial, proton carrier)
ULN	Upper limit of normal
UQCRC2	Ubiquinol-cytochrome c reductase core protein II
WES	Whole-exome sequencing
wt	Wild type
Δ pH	Mitochondrial transmembrane pH gradient
$\Delta\psi_m$ or $\Delta\psi$	Mitochondrial transmembrane potential
ρ 0 cells	Cell lines lacking mtDNA

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Chapter 1

General Introduction

1.1 Preface

This chapter briefly introduces the topics presented in this thesis, starting with a general overview of mitochondrial function. Various aspects of mitochondrial quality control such as mitochondrial dynamics, mitochondrial DNA maintenance, and protein folding will be emphasized. Literature review on high-throughput genome-wide screens will be presented, highlighting its utility in assigning novel function to genes. *TID1* and *ROMO1*, two novel genes that were uncovered from a genome screen will be reviewed. Lastly, the topics of *MFN2* and multiple symmetric lipomatosis will be discussed as it pertains to the final part of this body of work.

1.2 Mitochondrial Biology

1.2.1 Overview of Mitochondrial Function

Cellular metabolism regulates many aspects of human physiology. Muscle contraction, neurotransmitter release, development, and glucose-stimulated insulin release are examples of processes that are highly dependent on normal metabolic homeostasis [1-4]. Therefore it follows that mitochondria are also crucial in these processes because in most tissues, mitochondria are the sites of generation of large amounts of ATP, an energy-carrying molecule [5]. Mitochondria may use carbohydrates, fatty acids, or amino acids as substrates to generate energy depending on the cell type and substrate availability [6, 7]. Through glycolysis, glucose is broken down into pyruvate, which is transported into the mitochondrial matrix by mitochondrial pyruvate carriers (MPCs) [8, 9]. Palmitate, a commonly metabolized fatty acid, is transported into the matrix via the carnitine palmitoyltransferase system. Both pyruvate and palmitate are converted to acetyl-CoA, which drives the tricarboxylic acid cycle (TCA, also known as Krebs or citric acid cycle). Glutamine and glutamate are converted to α -ketoglutarate, which can also drive TCA cycle, in a process called anaplerosis [10]. NADH and FADH₂ produced by the TCA cycle donate electrons to the electron transport chain (ETC), which consists of four large, redox carriers (complex I – IV) (Fig 1.1). Electron transport in complexes I, III, and IV are tightly coupled to their proton pumping activity, which pumps protons from the matrix to the intermembrane space. Electrons are accepted by molecular oxygen at complex IV to produce water. The accumulation of protons in the intermembrane space against its electrochemical gradient generates a protonmotive force (PMF or Δp), which is used to drive ATP synthase (complex V), generating ATP. The loss of redox potential from one electron pair theoretically generates 2.5 ATP molecules.

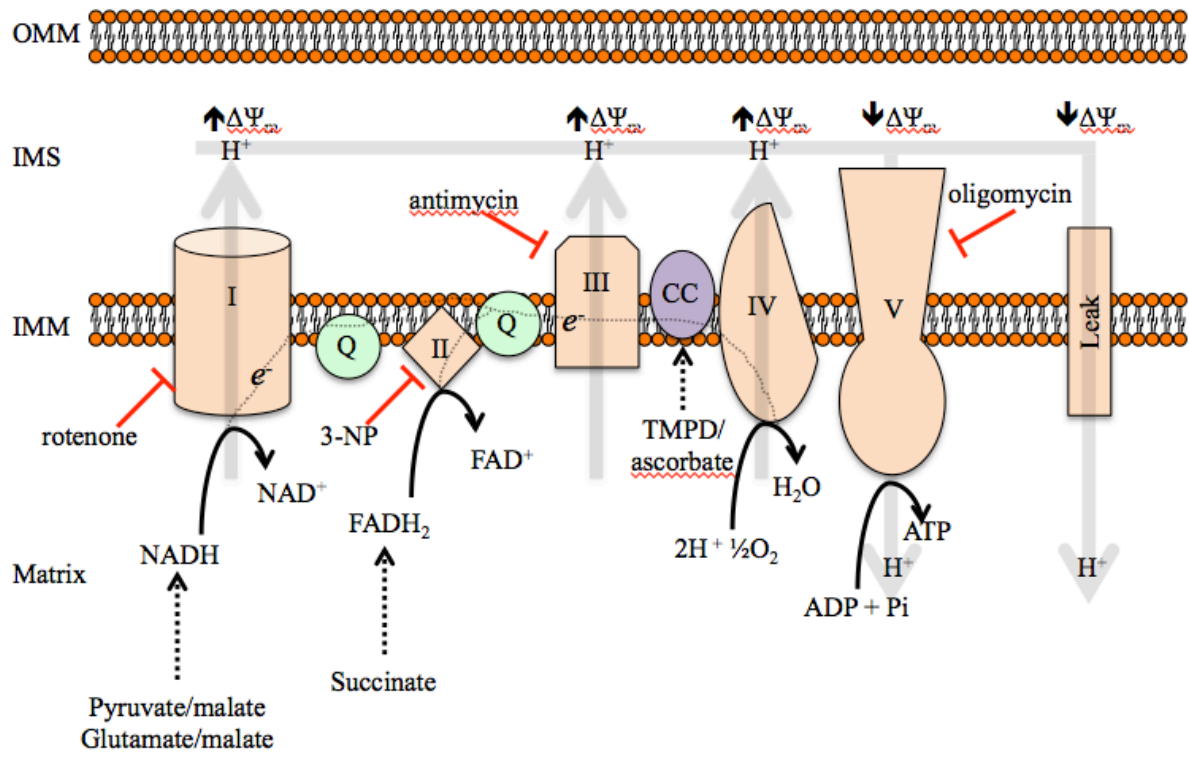


Fig 1.1: Schematic of electron transport chain. The four respiratory complexes (complexes I – IV), ATP synthase (complex V), and leak proteins are shown. Protons are pumped from the matrix into the intermembrane space by complexes I, III, and IV, increasing $\Delta\Psi_m$. Protons are shuttled back into the matrix through complex V and UCP, and in the process generating ATP and heat, respectively and decreasing $\Delta\Psi_m$. Several inhibitors of the respiratory complexes used in this study are listed (red inhibitory arrows). Direction of electron flow is shown in dotted lines. OMM = outer mitochondrial membrane, IMS = intermembrane space, IMM = inner mitochondrial membrane, Leak = leak proteins, H^+ = protons, $\Delta\Psi_m$ = mitochondrial membrane potential, NAD = nicotinamide adenine dinucleotide, $FADH_2$ = flavin adenine dinucleotide (hydroquinone form), TMPD = *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 3-NP = 3-nitropropionic acid, ADP = adenosine diphosphate; ATP = adenosine triphosphate; e^- = electron pair, Q = coenzyme Q, CC = cytochrome C, $\Delta\Psi_m$ mitochondrial membrane potential.

PMF (Δp) is comprised of a charge component ($\Delta\psi_m$) and a chemical component (ΔpH) and is represented by the following Nernstian equation: $\Delta p = \Delta\psi_m - 60\Delta pH$ [11]. Since typical values of Δp range from 180 – 220 mV, and ΔpH range from 0.5 – 1.0, $\Delta\psi_m$ accounts for most of Δp . Therefore, the driving force for ATP production is highly dependent on this charge gradient, or membrane potential. Furthermore, import of cytosolic protein precursors and some metabolites into the matrix and mitochondrial calcium ions sequestration are dependent on intact $\Delta\psi_m$ [12]. In addition, $\Delta\psi_m$ can modulate reactive oxygen species (ROS) formation [13]. While an increase in $\Delta\psi_m$ may be an indication of increase of substrate feed into ETC, and a decrease in $\Delta\psi_m$ may be a sign of mitochondrial dysfunction or ATP production, it must be noted that inhibition of ATP synthase (complex V) can theoretically and experimentally lead to an increase in $\Delta\psi_m$, and as such the value of $\Delta\psi_m$ is not a direct measure of “mitochondrial function”.

Mitochondria possess a ~16.6 kilobase pair circular genome that encodes 22 tRNAs, 2 rRNAs, and 13 of the ~80 - 100 ETC proteins. Mitochondria and nuclear-encoded ETC subunits are assembled in a coordinated fashion into functional respiratory complexes [14]. Various mitochondrial DNA (mtDNA) mutations in protein-encoding regions affect ETC integrity, mitochondrial respiration, and cause mitochondrial diseases such as Leigh disease and Leber Hereditary Optic Neuropathy (LHON) [15]. In addition, cells experimentally depleted of mtDNA (termed $\rho 0$ cells) are completely respiration-deficient, but can maintain an intact $\Delta\psi_m$, by the reversal of the F_1F_0 ATP synthase (complex V). The sacrifice of ATP to preserve $\Delta\psi_m$ highlights the importance of $\Delta\psi_m$ in maintaining processes such as cristae structure and protein and metabolite import [16]. Similar to nuclear genomic DNA, mtDNA associates with several proteins to form compact structures called nucleoids, allowing for

efficient genome maintenance, genome propagation and gene expression [17, 18]. Human mitochondrial transcription factor A (TFAM) is the most abundant protein found in nucleoids and is an indispensable factor for mtDNA packaging and nucleoid maintenance. Various fluorescent techniques have been used to visualize the mitochondrial nucleoid. These include DNA probes such as DAPI, Ethidium Bromide, BrdU and PicoGreen [19]. Antibodies against DNA and proteins associated with nucleoids have also been used [20]. These techniques confirm that mitochondrial nucleoids are organized as discrete structures along the mitochondrial network [21, 22].

1.2.2 Regulators and Effectors of Mitochondrial Dynamics

Pioneering biochemical and electron microscopy studies on isolated mitochondria have long forged the view that mitochondria existed and behaved as discrete, bean-shaped organelles [23]. Advances in fluorescent imaging techniques in the past three decades have confirmed that mitochondria, in fact, exist as an interconnected network sculpted by fission and fusion reactions, phenomena collectively known as mitochondrial dynamics (Fig 1.2). Mitochondrial fusion is a highly regulated process that involves the mixing of four membranes (outer membranes and inner membranes of two adjacent mitochondria) [24]. Mitochondrial fusion is mediated by transmembrane GTPases. In mammals, two mitofusins (MFN1 and MFN2) mediate outer membrane fusion [25]. OPA1 (Optic Atrophy 1) has a dual role in maintaining inner membrane fusion and integrity of cristae, inner membrane folds that increase surface area [26]. Mitochondrial fission occurs at sites of endoplasmic reticulum-mitochondrial (ER-mito) contact [27]. Mitochondrial fission factor (MFF) recruits cytosolic dynamin-related protein 1 (DRP1) to ER-mito contact sites to mediate fission [28]. Mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51, respectively) and

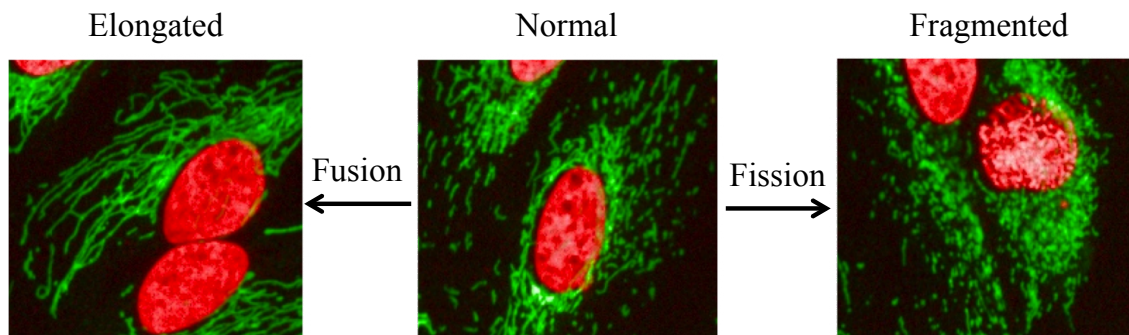


Fig 1.2: Mitochondrial fusion and fission. Fluorescence micrographs of cells stained for mitochondria (green) or nuclei (red). Mitochondria were stained using an antibody against TOMM20, an outer mitochondrial membrane protein. Hoechst 33342 was used to stain nuclei. Increase in fusion leads an elongated mitochondrial network, whereas increase in fission leads to a fragmented mitochondrial network.

mitochondrial fission 1 protein (FIS1) are other factors that can recruit DRP1 to mitochondria [29-31]. Once localized to the outer membrane, DRP1 proteins oligomerize and mediate membrane scission [32]. The exact mechanism of the membrane pinching is unclear, but may be analogous to endocytic membrane scission and involves its GTPase and GED activity [33-35]. DRP1 is extensively post-translationally modified. Phosphorylation of DRP1 at S637 by cAMP-dependent kinase (PKA) negatively regulates DRP1 [36, 37] and phosphorylation at S616 positively regulates DRP1 during mitosis [38, 39]. DRP1 activity is also affected by ubiquitination [40-42], sumoylation [43], and nitrosylation [44, 45]. Currently, no inner membrane fission mediator has been identified.

1.2.3 Mitochondrial Dynamics in Cell Biology

Interest in mitochondrial dynamics peaked when it was observed that upon treatment with a variety of apoptosis inducers, the typical tubular mitochondrial network is transformed into small, round organelles [46]. Mechanistically, this mitochondrial fragmentation is due to increased mitochondrial recruitment of DRP1 and loss of mitochondrial fusion from the collapse of $\Delta\psi_m$ [46-48]. Importantly, genetically or pharmacologically blockage of mitochondrial fragmentation significantly delays cytochrome C release, caspase activation, and downstream cell death [28, 46, 49, 50], whereas increasing fragmentation has the opposite effect [49].

Mitochondrial dynamics plays a central role in mitochondrial quality control. Mitochondrial fission separates healthy mitochondria from damaged, $\Delta\psi_m$ -collapsed mitochondria. Because fusion requires $\Delta\psi_m$, damaged mitochondria are unable to re-fuse [48]. These mitochondria are then engulfed by autophagosomes and eliminated in a process called mitophagy,

allowing the cell to maintain a functional complement of mitochondria [51]. Genetic inhibition of mitochondrial fission attenuates mitophagy and leads to an accumulation of oxidized, damaged proteins [51, 52]. In brown adipocytes, a cell type rich in mitochondria, norepinephrine induces dramatic DRP1-dependent mitochondrial fragmentation [53]. This facilitates free fatty acid (FFA)-induced uncoupling of mitochondria and leads to a physiological increase in energy expenditure and thermogenesis.

Mitochondrial morphology varies in different stages of the cell cycle [54]. During mitosis, mitochondrial network can dramatically fragment into many individual organelles to facilitate mitochondrial segregation to daughter cells. Interestingly, mitochondria undergo hyperfusion during the G1-S transition, characterized by a formation of giant network. Hyperfused mitochondria promote cyclin E buildup and coordinate the transition from G1 to S phase.

1.2.4 Role of Mitochondrial Dynamics in Human Physiology and Diseases

Genes encoding mitochondrial dynamics proteins are widely expressed in various tissues [55, 56]. Murine knockouts of *Mfn1*, *Mfn2*, *Opal*, and *Drp1* are embryonic lethal, highlighting the essential role of mitochondrial dynamics in development [57-60]. In humans, mutations in *MFN2* cause Charcot-Marie-Tooth disease. *OPA1* mutations cause dominant optic atrophy [61, 62] and may be associated with other neurological abnormalities such as hyperreflexia, ataxia, and sensorineural hearing loss [63, 64]. The only reported human mutation in *DRP1* caused lethal microencephaly, optic atrophy, and lactic acidemia in a newborn girl [65].

Altered mitochondrial dynamics is also implicated in age-related diseases. Brain tumor initiating cells (BTICs) have increased mitochondrial fragmentation and DRP1 activity relative to non-BTICs [66]. Similarly RAS-mediated oncogenic transformation requires

DRP1 activation, leading to mitochondrial fragmentation [67]. In addition, reducing DRP1 activity genetically or pharmacologically in various cancer cell-types reduced cell proliferation and/or increased apoptosis in various cancer cell types [68]. In age-related neurodegenerative diseases such as Alzheimer disease and Huntington disease, neuronal mitochondria show increased fragmentation due to increased DRP1 activity [45, 69]. Increased mitochondrial fragmentation has been observed in pancreatic beta cells, skeletal muscle cells, pulmonary arterial hypertension, and endothelium of individuals with Type 2 diabetes [70-72]. Given the relevance of dysregulated mitochondrial fission and fusion in various inherited and age-related diseases, research on mitochondrial dynamics has intensified over the past three decades. However, the precise mechanisms regulating mitochondrial dynamics are still not fully elucidated [73]. What are the signals that the mitochondrial reticulum responds to? How do changes in mitochondrial dynamics affect disease outcomes? *The identification of novel regulators of mitochondrial dynamics and the characterization of these novel genes are major topics of the present research.*

1.3 High-throughput Genetic Screens

1.3.1 High-throughput Screens to Assign Novel Functions to Genes

Ever since the complete sequencing of the human genome, functional identification of all identified genes has been a bottleneck in cell biology [74]. In 1998, Mello and Fire's Nobel Prize discovery of the mechanism of RNA interference (RNAi) allowed for simple, loss of function studies of unknown genes [75]. It was later shown that ~20 nucleotide double-stranded RNA fragments activate RNAi-induced silencing complex (RISC), and post-transcriptional gene silencing [76]. These small interfering RNAs (siRNAs) can be chemically synthesized and mediate RNAi in cultured mammalian cells [77]. Development

of arrayed siRNA libraries in 96-well and 384-well formats allowed for systematic loss-of-function studies. Along with siRNA libraries, various vendors have also developed overexpression arrays for gain of function screens. Genome-wide RNAi screens have been used to identify genes involved in growth and viability [74], polyglutamine aggregation [78], innate immunity [79, 80], longevity [81], and various signaling pathways [82-85]. Using genome-wide overexpression screen, we have identified Transducers of Regulated CREB activity (TORCs/CRTCs) that mediate cAMP response element binding protein (CREB) target gene expression [86, 87]. We subsequently showed that glucose, calcium and cAMP control CRTC2 activity to mediate hepatic gluconeogenesis and modulate pancreatic insulin secretion [87-89]. Together, this body of work shows the utility of genome-wide screens in assigning function, paving the way for the physiological understanding of these novel genes.

1.3.2 Design and Optimization of a High-throughput Screen

The miniaturization of the screening assay is critical to executing a successful high-throughput screen. This step, however, is not trivial. For example, miniaturization of nucleic acid transfection and cell culture conditions from large well to small well format requires extensive empirical testing. The functional assays used must also be amenable to high-throughput screening. Automated liquid dispensers, liquid handlers, and robotic equipment should be tested and used during the optimization phase. Also, positive controls need to be identified and tested in order to determine the screenability of the assay. *In this research, the development, optimization, and execution of a high-throughput imaging screen will be used to identify novel genes required for normal mitochondrial morphology.*

1.4 Identification of *TID1* and *ROMO1* as Novel Critical Regulators of Mitochondrial Quality Control

Below, I will present a brief review of two genes that were identified in a genome-wide screen for novel regulators of mitochondrial dynamics.

1.4.1 Role of *TID1* in Cell Biology and Physiology

Tumorous imaginal discs 1 (*TID1/DNAJA3*) gene encodes two mitochondria-localized proteins (TID1-S and TID1-L) that differ in their C-termini through alternative splicing [90]. TID1 possesses an N-terminal J domain and is therefore classified as a DNAJ/HSP40 family of molecular chaperones. HSP40 proteins act as co-chaperones that stimulate the ATPase activities of HSP70 proteins. TID1 is proposed to function with mortalin/HSPA9/mtHSP70 as its co-chaperone since mortalin is the only known HSP70 that is localized to mitochondria. *In vitro* analyses using cell-free systems indeed show that TID1 activates mortalin to disaggregate misfolded proteins [91]. Previous work has shown a role for TID1 in regulating apoptosis, interferon signaling, embryonic development, neurite outgrowth, neuromuscular junction signaling, and preventing dilated cardiomyopathy (Table 1.1) [90, 92-96]. Conceptually, TID1 may modulate these cytosolic signaling pathways while transiting from cytosolic ribosomes to mitochondria by interacting with cytosolic HSP70s [97]. Data from biochemical and cell biology studies show that TID1 localizes overwhelmingly to mitochondria, and as such, it is surprising that a mitochondrial role for TID1 has been elusive. *The characterization of the mitochondrial role of TID1 is a major topic of this thesis.*

1.4.2 Role of *ROMO1* in Cell Biology

Reactive oxygen species modulator 1 (*ROMO1/MTGM/c20orf52*) gene encodes a mitochondria-localized protein and is upregulated in various cancer tissues [98-100]. *ROMO1*-silencing inhibits the growth of various cancer cell lines [101]. Overexpression of ROMO1-myc led to increased mitochondrial superoxide, cellular reactive oxygen species

Function	Species	References
mtDNA and folding of in vitro synthesized protein	Yeast	Rowley et al, <i>Cell</i> , 1994. Duchniewicz et al <i>MCB</i> , 1999.
Folding of in vitro synthesized protein	In vitro yeast mitochondria	Westermann et al, <i>MCB</i> , 1996. Prip-Buus et al, <i>FEBS Letters</i> , 1996.
Interaction with HPV E7 protein	Human	Schilling et al, <i>Virology</i> , 1998
Apoptosis	Human	Syken et al. <i>PNAS</i> , 1999. Syken et al. <i>Oncogene</i> , 2003. Edwards et al. <i>Oncogene</i> , 2004. Trentin et al. <i>FEBS Letters</i> , 2004.
Interferon signaling Pathway	Human	Sarkar et al. <i>JBC</i> , 2001.
Embryogenesis	Mouse (animal)	Lo et al, <i>MCB</i> , 2004.
Neurite outgrowth	Human	Liu et al, <i>JBC</i> , 2005.
Cardiac function	Mouse (animal)	Hayashi et al, <i>Nature Medicine</i> , 2006.
Neuromuscular function	Mouse	Linnoila et al, <i>Neuron</i> , 2008.
p53 mitochondrial localization	Human	Ahn et al, <i>Oncogene</i> , 2010
Parkinson Disease (6-OHDA Model)	Rat	Proft et al, <i>Plos One</i> , 2011
Folding of aggregated protein in vitro	In vitro Recombinant human proteins	Iosefson et al, <i>Cell Stress and Chaperones</i> , 2012.

Table 1.1: Known cellular roles of TID1. Previous known functions of human TID1 and its yeast and mouse homologs. Most research focused on various cytosolic signaling roles of TID1. However, a mitochondrial role for TID1 remains to be clarified.

(ROS) as determined by fluorescent cellular ROS detector dichlorofluorescein diacetate (DCF-DA), and decreased $\Delta\psi_m$ [98]. While it is possible that these data suggest a specific role of ROMO1 in generating ROS, the parsimonious explanation of the above observations is non-specific mitochondrial dysfunction perhaps due to inner membrane protein overload [19]. In addition, the Nister group used a C-terminal tagged ROMO1 construct to show a role of ROMO1 in promoting mitochondrial fission and fragmentation [102]. However, in our hands, we showed that C-terminal tagging of ROMO1 phenocopied the effects of ROMO1-silencing, suggesting that this construct acted as a dominant negative mutant [103]. Furthermore, we used siRNA and N-terminal and untagged rescue constructs to show that ROMO1 *prevented* the fragmentation of mitochondria by modulating the activity of OPA1, a protein involved in inner membrane fusion. *ROMO1*-silencing led to a loss of OPA1 oligomers and increased processing of long OPA1 (OPA1-L) to short OPA1 (OPA1-S) isoforms (Fig 1.3). A *Romo1* knockout mouse has not yet been reported, and thus the physiological role of *Romo1* remains unclear.

1.5 MFN2 Mutation as a Cause for Multiple Symmetric Lipomatosis

The second part of my thesis implicates the mitochondrial fusion gene *MFN2* in the pathogenesis of a disease called multiple symmetric lipomatosis. Below, I review the current literature pertaining to the *MFN2* gene

1.5.1 Role of MFN2 in Mitochondrial Fusion

Human mitofusins 1 and 2 (MFN1 and MFN2) are homologs of *Drosophila melanogaster* fuzzy onions (Fzo) proteins [25]. Similar to MFN1 and MFN2, Fzo protein is a large transmembrane GTPase. *Fzo* mutants resulted in defective Nebenkern mitochondrial fusion during spermatogenesis [104]. Electron transmission microscopy on mutant Nebenkerns

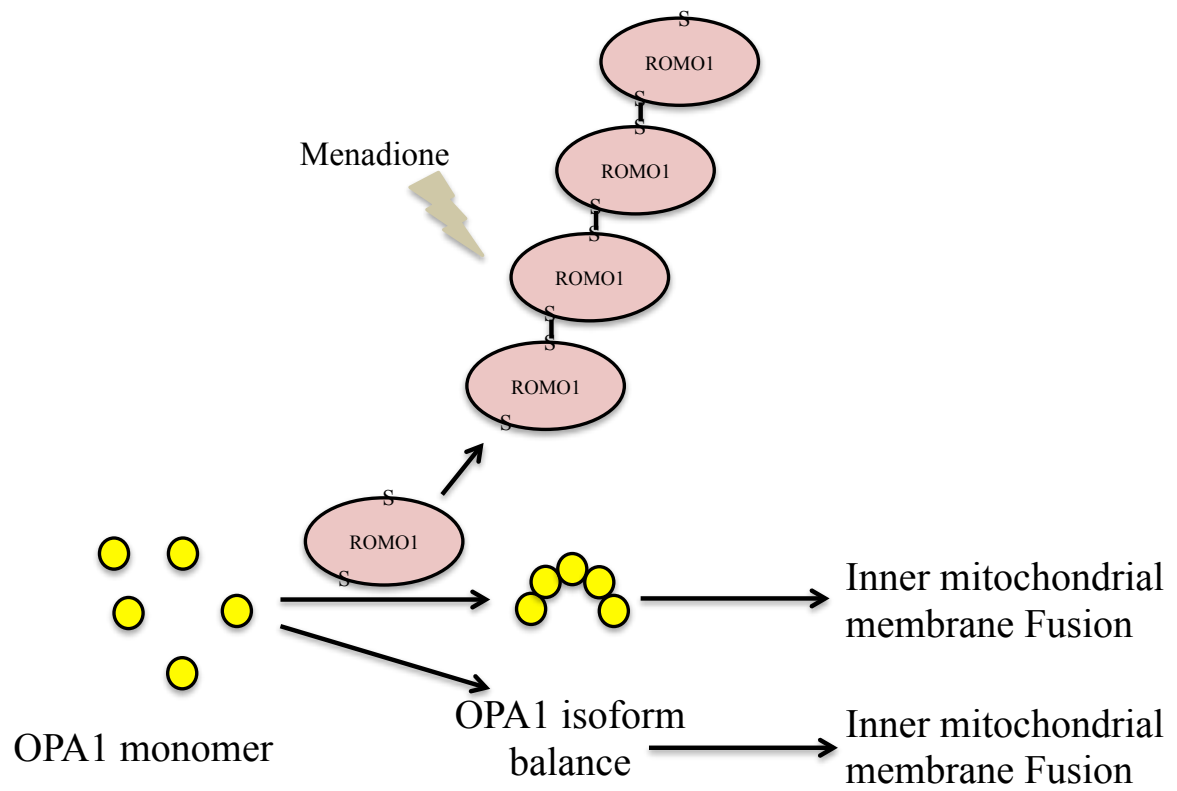


Fig 1.3: ROMO1 is a redox-sensitive protein involved in mitochondrial dynamics. In response to the redox cyler manadione, ROMO1 is oxidized inactivated and forms disulphide bridges. Monomeric ROMO1 maintains OPA1 oligomers and maintain OPA1 isoform balance, both required for mitochondrial inner mitochondrial membrane fusion.

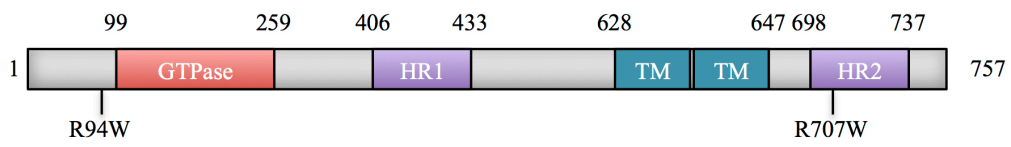
revealed the appearance of smaller onion-shaped mitochondria that appeared to wrap around each other, and hence the designation “*fuzzy onions*”. MFN1 and MFN2 proteins are expressed in all tissues examined to date, but differ in the relative amounts in each tissue [55, 105]. For example, MFN2 is expressed at a much higher level than MFN1 in brain. In contrast, heart tissue preferentially expresses MFN1.

MFN2 mitochondrial fusion activity depends on its N-terminal GTPase activity (Fig 1.4) [25]. Mitofusins contain two heptad-repeat domains (HR1 and HR2) that flank a bipartite transmembrane domain. Crystal structure reveals that the C-terminal HR2 form a dimeric antiparallel coiled-coiled structures that mediate mitochondrial outer membrane tethering [106]. Transient expression of high levels of MFN2 results in perinuclear mitochondrial clustering that is dependent on the C-terminal 54 amino acids [25]. Immunoprecipitation experiments show that MFN1 and MFN2 can form homo- or hetero-oligomeric complexes [57, 105]. MFN2 may also play a role in maintaining endoplasmic reticulum-mitochondria contact sites [107].

1.5.2 *MFN2* Mutations Cause CMT2A

Charcot-Marie-Tooth (CMT) disease (also known as hereditary motor and sensory neuropathy/HMSN) is an inherited neurological disorder characterized by sensory loss and muscle atrophy in distal limbs [108]. CMT is divided into two main types based on pathological and clinical findings. CMT Type 1 (CMT1) is a demyelinating disease and therefore decreases nerve conduction velocities (NCV) [109]. In contrast, CMT Type 2 is characterized by axonopathy with normal NCV. CMT disease is further subdivided based on genetic causes. Autosomal dominant *MFN2* mutation is causal for most types of CMT2 disease (CMT2A) [110]. MFN2 Arg94 in the GTPase domain is a hot spot for CMT2A

A



B

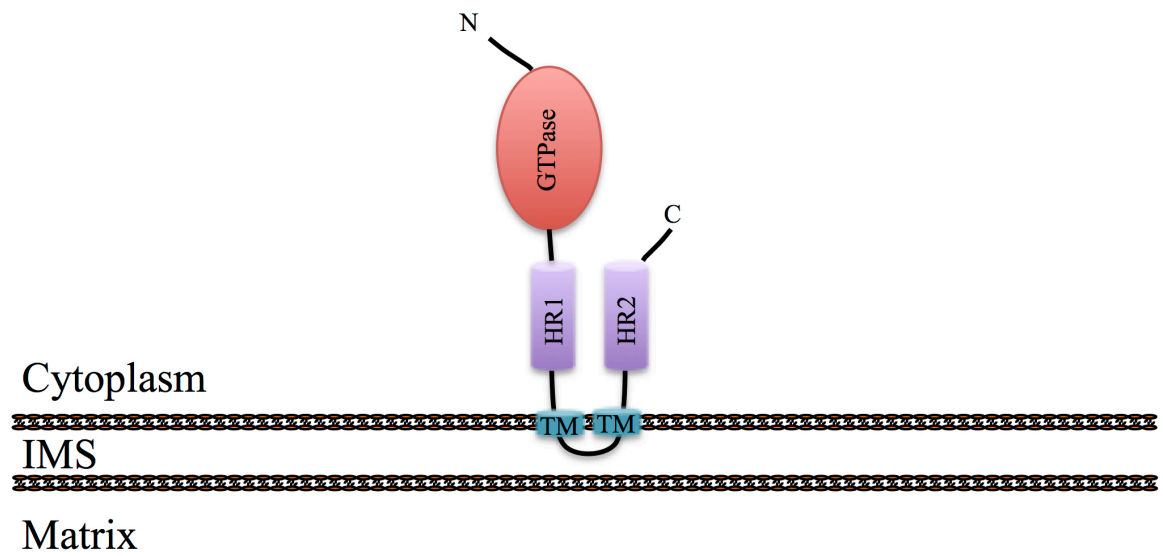


Fig 1.4: Schematic of MFN2 protein. (A) Domain structure of MFN2 protein. MFN2 contains a N-terminal GTPase domain where most CMT2-causing mutations occur. HR1 and HR2 domains can form coiled-coiled structures. TM domain is bipartite in nature, allowing MFN2 protein to pass the outer membrane twice. Arg707 residue is located in the HR2 domain. The numberings refer to the positions of the amino acid residues. HR1 = heptad repeat 1 domain, HR2 = heptad repeat 2 domain, TM = transmembrane domain. (B) Orientation of MFN2 with respect to the cytoplasm and the mitochondrial outer membrane. IMS = intermembrane space

mutations and was found to be mutated to tryptophan or glutamine in several patient series, resulting in severe CMT [111, 112].

1.5.3 Multiple Symmetric Lipomatosis/Madelung's disease as a mitochondrial disease of brown fat origin

Multiple Symmetric Lipomatosis (MSL, also known as Benign Symmetrical Lipomatosis, and Madelung's Disease) is a rare cutaneous disorder characterized by the symmetrical accumulation of non-encapsulated fat deposits in the neck, shoulder, upper trunk, and proximal upper limbs (Fig 1.5). 42 years after Brodie's original description of the condition in two patients [113], Madelung gave detailed phenotypic descriptions of three patients with fat deposits in the submental regions [114]. The etiology and exact incidence of MSL is unknown, but appears to preferentially affect males of Mediterranean and Eastern European descent. Enzi estimated that MSL occurs with an incidence of 1 in 25,000 males in Italy [115]. Excessive consumption of alcohol appears to be a risk factor for MSL [116]. The disorder is usually diagnosed during middle age and is slowly progressive [114]. Surgical removal of lipomas is the treatment of choice for those with severe cosmetic concerns and upper airway compression [117-121]. It is hypothesized that MSL is a mitochondrial disorder with a brown fat origin [122]. In support of this, the distribution of lipomas in MSL resembles that of brown fat, a tissue thought to be present at low amounts in the human adult [123]. Some surgically resected lipomas from MSL patients tested positive for UCP1 expression, a specific marker for brown adipocytes [124, 125]. In addition, there is clinical-pathological association with myoclonic epilepsy and ragged red fibers (MERRF) syndrome, a mitochondrial disease characterized by myoclonus, seizures, ataxia, and myopathy [126, 127]. Skeletal muscle biopsies from MSL

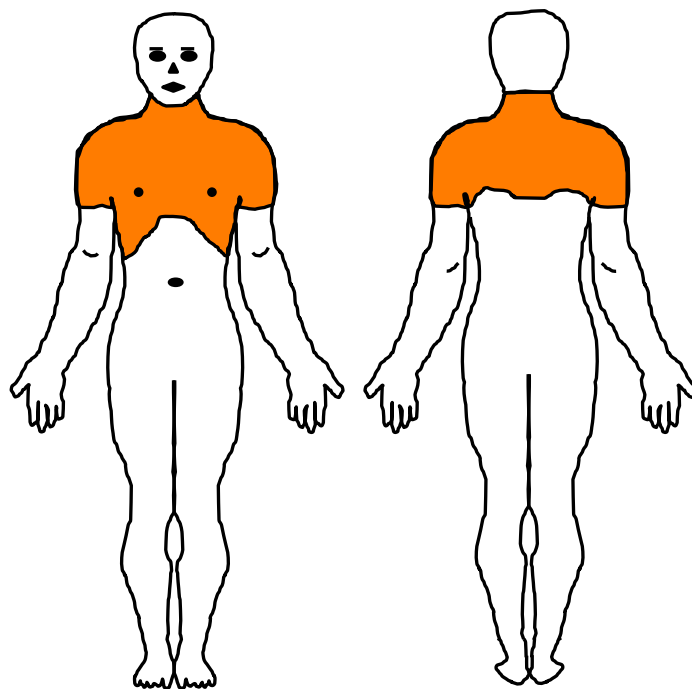


Fig 1.5: Areas affected in individuals with multiple symmetric lipomatosis (MSL).

Ventral (left) and dorsal (right) views of human cartoon are shown. Orange-shaded areas are typical areas where lipomas accumulate in patients with MSL. Figure adapted from [128]

patients show mtDNA depletion and decreased cytochrome C oxidase activity, hallmarks of mitochondrial dysfunction [129, 130]. Finally, the pathological mtDNA tRNA(Lys) A8344G mutation has been found in MSL patients [127, 131]. Taken together, these findings suggest that MSL is caused by mitochondrial dysfunction and may have a brown fat origin.

1.6 Hypotheses and Research Objectives

My thesis research project is divided into two parts.

Part 1: Identification of Novel Regulators of Mitochondrial Dynamics

While the effectors of mitochondrial fission and fusion, collectively known as mitochondrial dynamics, is a subject of intense research, the regulators of mitochondrial dynamics are still relatively unknown. Therefore, our working hypothesis is as follows:

High throughput loss-of-function screens are useful to identify novel regulators of mitochondrial quality control.

Specific objectives:

1. Identify novel regulators of mitochondrial dynamics using high-throughput genome-wide screening.
2. Identify and characterize the role of *TIDI* in mitochondria.
3. Determine the mechanism that leads to the loss of maximal respiration in *ROMO1*-silenced cells.

Part 2: Homozygous Mutations in *MFN2* Cause Multiple Symmetric Lipomatosis with Neuropathy

Using whole-exome sequencing, Drs. Sawyer and Boycott's team identified a homozygous variant c.2119C>T;p.R707W in two brothers with multiple symmetric lipomatosis (MSL). Therefore, our working hypothesis is as follows:

MFN2 R707W mutation impairs its ability to mediate mitochondrial fusion.

Specific objectives:

1. To identify and characterize any functional impairment caused by the MFN2 R707W mutation and compare results with the severe CMT mutant, R94W.
2. To provide evidence that MSL has a brown fat origin.

1.7 References

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Chapter 2

High-Content Functional Genomic Screening to Identify Novel Regulators of the PINK1-Parkin Pathway

2.1 Preface

I designed and optimized a high-throughput protocol to screen for novel genes required for regulators of mitochondrial dynamics. This screen successfully identified a gene called *ROMO1* in mediating mitochondrial fusion. Valerie Lefebvre (former masters student) used this protocol to screen for genes involved in *PINK1-PARK2* pathway and identified a critical role of *ATPIF1* and ATP synthase in mediating uncoupler-induced mitochondrial recruitment of PARK2 (Appendix 1). Subsequently we were invited to write a methods paper, presented in this chapter, describing our *PINK1-PARK2* screen. This methods paper, published in *Methods in Enzymology*, contained other important general considerations when designing high-throughput screens.

Author list

Andy Cheuk-Him Ng, Stephen D. Baird, Robert A. Screaton

Author contribution

AC-HN designed and optimized the screen protocol outlined in this chapter. SDB provided ideas and wrote the computer algorithmic script, which was used in the automated microscopic detection of mitochondria. RAS provided ideas and supervised the study. AC-HN wrote the first draft of this paper. All authors participated in revision process.

2.2 Abstract

PINK1/PARK6 and Parkin/PARK2 are amongst the most commonly mutated genes associated with recessive forms of familial Parkinson disease (PD). Recent evidence indicates that the proteins they encode, PINK1 and Parkin, function in the same pathway to mediate the selective autophagic clearance of dysfunctional mitochondria. Upon mitochondrial damage, PINK1 is stabilized on the mitochondrial outer membrane where it phosphorylates ubiquitin, generating a signal for the recruitment and activation of Parkin. However, key mechanistic questions still exist regarding Parkin recruitment, including whether or not other factors are required for the PINK1 and Parkin pathway. We describe a method below using high throughput RNA interference technology to interrogate the genome for novel components of the PINK1 and Parkin pathway.

2.3 Introduction and Theory

Mutations in PINK1/PARK6 and Parkin/PARK2 are associated with familial forms of PD [1, 2]. Parkin has been shown to be neuroprotective in various models [3-5]. The seminal discovery that Parkin is recruited to depolarized mitochondria to initiate its autophagic clearance suggested that Parkin could be involved in mitochondrial quality control [6]. This finding has now been confirmed by several groups in cultured cancer cell lines [7-9] and in neurons, albeit with altered kinetics [10-12].

Loss of mitochondrial membrane potential following treatment with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or the ionophore valinomycin leads to the accumulation of PINK1 kinase on the outer mitochondrial membrane [13, 14], where it phosphorylates Parkin at Ser65 and ubiquitin at Ser65 to stimulate Parkin mitochondrial translocation and E3 ligase activity [15-18]. Activated Parkin ubiquitinates outer mitochondrial membrane proteins, including mitofusins (MFN1/2) and RHOT1/2 to facilitate mitochondrial fission and downstream mitophagy [19-22].

RNA interference technology has been widely used as a tool to assign genes to signal transduction pathways based on their function [23]. The development of commercially available genome-scale libraries of siRNA and shRNA has enabled investigators to ask questions at a systems level. Genome-wide RNA interference screens are powerful approaches that permit unbiased identification of new genes involved in various aspects of immunology, cancer, and ageing [24-30]. With advancements in the molecular science of RNAi targeting, improved RNAi sequence prediction tools have enabled the generation of more effective siRNA libraries that minimize off-target effects [30, 31].

We and others have performed genome-wide RNAi screens to look for novel regulators of PINK1 and Parkin pathway [27, 32]. Specifically, we screened for genes that blocked CCCP-induced Parkin mitochondrial translocation after they had been silenced with siRNA. We identified ATP1F1 (or IF1), an endogenous inhibitor of the reverse (or ATPase) activity of the F1Fo ATP synthase, as a critical component of depolarization-induced Parkin-dependent mitophagy [27]. Loss of IF1 blocked the collapse of delta psi and subsequent accumulation of PINK1, indicating that IF1 and the ATP synthase are potential targets for modulating Parkin-dependent mitophagy.

2.4 General Screen Design Strategy

Here we describe the central considerations in the design of a high-throughput RNAi screen. There are three main steps: Assay design and miniaturization, executing the screen, and follow-up validation. Careful assay design, with a particular focus on protocol simplicity allows for the generation of a focused candidate list and reduced effort in the more time-consuming validation stage.

2.4.1 Biological Systems

When choosing a biological system for screening, one must appreciate that practicality must trump biological elegance. For example, while primary cells may be used because of their similarities to *in vivo* tissues, immortalized cell lines offer numerous practical advantages such as transfectability and ease of propagation. Table 2.1 contains some of the points to consider when choosing a biological system for use in a screen.

Biological Systems

Immortalized Cell line	Primary Cell line
High transfectability	Good to use if cell type is important and immortalized line is unavailable
Good to use if general cell biology question is to be probed	May share more characteristics to tissues <i>in vivo</i>
Unlimited regenerative potential	

Table 2.1: Comparison of the two general types of biological systems commonly used for high-throughput screening.

2.4.2 Screen Libraries

Table 2.2 lists the most common libraries being used for high-throughput screens. siRNA libraries are widely used due in part to their high transfectability. This trait is required for assays that measure parameters of every cell screened such as high content or imaging based-assays. However, we and others have experienced widespread off target effects using these libraries, so considerable follow-up experiments are needed to verify hits [33, 34]. cDNA libraries can be used if the parameter being measured can be assigned on a cell to cell basis, thus for fluorescence assays, or for luminescence and absorbance assays if the signal is sufficiently strong assuming the variation in transfection efficiencies from well to well can be effectively minimized [26].

2.4.3 Choice of Assays

A critical point is to design an assay with a high signal to noise ratio to provide a wide separation between controls. This is particularly important when considering that many relevant targets may only be partially silenced with the library RNAi. It is preferable if not an absolute requirement to design an assay with minimal steps or interventions so as to be amenable to high throughput protocols. Table 2.3 lists several points to consider when designing a high-throughput assay. Measuring a parameter that is too far downstream in a given pathway may generate a large list of candidates removed from the specific biological event being studied. For example, if the goal is to find novel components of the PINK1-Parkin pathway, it is ill-advised to monitor mitochondrial mass, for example using intensity of TOMM20 immunofluorescent signal, as an output. Performing such a screen will yield a

Commonly Used libraries

	siRNA	cDNA libraries	Compound Libraries
Transfectability	High	Low to moderate	N/A
Off-targets	Widespread	Overexpression artifacts	Widespread
Critical follow-up experiments	Correlate knockdown efficiency with phenotype using multiple siRNAs, genetic rescue experiments		Deplete target genetically and show effect of compound is lost
Advantages	Basic understanding of biology probed	Basic understanding of biology probed	Closer to translational research

Table 2.2: Commonly used libraries used for high-throughput screening.

Screening assay

Arrayed or pooled libraries?

Luminescence, absorbance, microscopy based?

Signal to noise? Positive Controls? Z' factors?

Single or multiple timepoints?

How relevant is the assay to the specific biological question asked?

Table 2.3: Additional points to considered when designing a screening assay.

candidate list that will include genes involved in mitochondrial biogenesis, autophagy, mitochondrial dynamics, and cell proliferation.

The “screenability” or robustness of a high-throughput screen can be expressed as a Z' factor [35], which is calculated as follows:

$$Z' = 1 - \frac{3\sigma(\text{pos control}) + 3\sigma(\text{neg control})}{|\text{mean}(\text{pos control}) - \text{mean}(\text{neg control})|}$$

where σ is the standard deviation of the indicated controls. Briefly, its value expresses how effectively the assay separates positive and negative control values. From the equation, one can see that the Z' factor is based on the means of the control values and their standard deviations. A value of 1 is the theoretical ideal value. A screening assay that has a Z' factor of 0.5-1.0 is an excellent screen, predicted to identify hits; a screen with a Z' factor < 0.5 or even below 0 will likely generate larger lists of candidates with mild phenotype that are difficult to distinguish from controls and require excessive follow up validation.

2.4.4 Follow-Up Validation

Because siRNA can result in the downregulation of numerous targets due to unwanted off-target effects, it is therefore important to verify that the phenotype is caused by silencing the specific gene of interest. Achieving the same functional effect with two (or more) independent siRNA sequences targeting different regions of the candidate genes' transcript is a strong indication of an on-target effect. A *bona fide* hit will show a direct correlation between knockdown efficiency by western blotting or quantitative polymerase chain reaction

(QPCR) and functional effect in the screen assay. However, the gold-standard validation approach is to perform genetic rescue experiments wherein the control phenotype is restored by introduction of an siRNA-resistant cDNA construct in the presence of the functional siRNA. For a variety of reasons, rescue experiments are often very challenging, and sometimes not feasible. For a cell-based imaging screen, the ideal rescue conditions consist of restoring the targeted protein to endogenous levels in each cell in the population. However, the use of plasmid transfection or viral vectors often lead to supraphysiological accumulation of protein product in only a subset of cells in the population. This may cause non-specific cell stress, protein/organelle aggregation, or dominant negative effects. However, we find that this can be overcome by using attenuated viral promoters that result in more physiological and homogeneous expression levels across the entire assay population [36].

2.5 RNAi Screen for genes required for PARK2 translocation

Below we describe the genome screen we performed to elucidate regulators of Parkin translocation [27].

2.5.1 Assay Design

In order to directly determine the factors required for PINK1-Parkin activation, we screened for genes that blocked mitochondrial translocation of exogenously introduced green fluorescent protein (GFP)-tagged Parkin in HeLa cells. We used the protonophore CCCP to depolarize mitochondria and to trigger mitochondrial translocation of Parkin. Cells are then fixed and high throughput immunofluorescence was performed to stain mitochondria. A colocalization algorithm from the Cellomics Arrayscan V^{TI} software was optimized to detect the degree of colocalization between GFP-Parkin and mitochondrial signals.

We have found that 384 well plates provided a screenable genome-wide platform. Each screen plate contained as a positive control, siRNAs targeting PINK1, and non-targeting negative control siRNAs to track whether differences in detection exists from one plate to the next. The siGENOME library set from Dharmacon was reconstituted as 80 nM stocks and 5 uL was arrayed into each well. For 18,000 siRNAs in the genome set, sixty-six 384-well plates were used for each replicate. The screen was performed in triplicate.

2.5.2 Execution of Genome-Wide Screen

The screen was performed with 66 384-well plates in triplicate, or 198 screening plates total. Plates in which test siRNA has been arrayed along with negative (non targeting siRNA) and positive (PINK1 siRNA) controls were thawed out on day 0 and reverse transfected into low-passage HeLa cells. At the same time purified adeno-associated virus (AAV) expressing GFP-Parkin was added to the cell culture medium. After a 72 h incubation to achieve knockdown, CCCP was added to each well for 1 h to stimulate recruitment of GFP-Parkin to mitochondria. Cells were then fixed and subjected to a high-throughput immunofluorescence protocol. All of the above steps were performed with high-throughput dispensers and liquid-handling equipment. Stained plates were then imaged using a Cellomics Arrayscan V^{TI} robotic imager, and data analysis performed with a colocalization algorithm we adapted from Cellomics vHCS Scan software. The candidate hit list, based on the degree of absence of colocalization of GFP-Parkin with mitochondrial signal, was generated with the Cell HTS2 Bioconductor software package [37].

2.5.3 Follow-Up Validation

Genes that displayed the least colocalization of GFP-Parkin and mitochondrial signals were sorted to the top of the candidate list. The PINK1 siRNA positive control and PINK1 siRNA pool from the genome library were found at the top of the candidate list with the highest Z score, indicating that the screen was well-executed. Given the known off-target effects of RNAi, it was essential to perform validation in order to pick out actual hits. Deconvoluted reagents to the top candidates were ordered and the Parkin-translocation assay was repeated with these reagents. Genes with 2 or 3 individual siRNAs that block Parkin recruitment were deemed high-confidence candidates, of which ATP1F1 was the strongest hit. We performed genetic rescue experiments with both a wt ATP1F1 and a non-functional mutant cDNA to rule-out off-target effects [27].

2.6 Equipment

- KiNEDx-660 Robot and Rail (Peak Robotics),
- Plate Depot Stack Carousel model CS-RS-450 (Peak Robotics),
- Cellomics ArrayScan V^{TI} HCS Reader (Thermo Scientific),
- 384-Well tissue-culture (TC) Microplates (BD Falcon OptiPlex cat. 353962),
- ELx405 Select CW cell washer (BioTek),
- Multidrop Combi reagent dispenser (Thermo Scientific),
- Janus liquid handler with four varispan tips and 384 MDT head (Perkin Elmer),
- Alps 3000 plate sealer (Thermo Scientific),
- Genera Automation and scheduling software (Retisoft Inc.).

Figure 2.1 shows the automated robotics set up in our robotics facility. Screen plates are loaded into the plate depot, which is placed near the ArrayScan V^{TI} microscope. The kiNEDx

robot arm is programmed to transfer screen plates from the plate depot to the microscope. Once a screen plate is scanned, the robot arm is programmed to transfer the plate to a column of the plate depot containing finished screen plates. The microscope is placed on a gas-cushioned bench to absorb any vibrations that may affect imaging, or alter the positioning of the microscope.

2.7 Materials

- Human siRNA libraries (siGENOME set from Dharmacon),
- SMARTpool: siGENOME PINK1 siRNA (cat. M-004030-02-0010; Dharmacon),
- Negative control siRNA (20 nmol) (cat. 1027310; Qiagen),
- Lipofectamine RNAiMAX (cat. 13778150; Life Technologies),
- OptiMEM® reduced serum medium powder (cat. 22600-050; Life Technologies),
- Sodium bicarbonate (NaHCO₃) (cat. S5761; Sigma Aldrich),
- DMEM 4 mM L-Glutamine, 4500 mg/L Glucose (cat. SH30022.01; HyClone)
- 10,000 U/mL Penicillin-10,000 ug/mL streptomycin (cat. SV30010; Hyclone),
- FBS (cat. F1051; Sigma-Aldrich),
- Bovine Serum Albumin (BSA) (cat. A2153-100G; Sigma-Aldrich),
- 500 mL 0.22 uM PES filter system (cat. 431097; Corning),
- RNase-free water (cat. W4502; Sigma-Aldrich),
- Rabbit polyclonal IgG anti-TOMM20 (cat. sc-11415; Santa Cruz Biotechnology),
- AlexaFluor® 594 Goat Anti-Rabbit IgG (H+L) (cat. A-11012; Life Technologies),
- Plastic plate seals (cat. 361006007; Fisher Scientific).



Fig 2.1: High-throughput screening facility. Equipment is positioned to allow KiNEDx robotic arm to transfer screen plates from the plate stacker to the Cellomics ArrayScan V^{TI} microscope.

2.7.1 Solutions and Buffers

1. Resuspend siRNA library to 2 μ M in siRNA Storage Buffer (Dharmacon) and from that rearray into 384-deep-well plates at a working concentration of 80 nM in nuclease-free ddH₂O. Using liquid handler and 384-multi-tip MDT head, daughter 5 μ L per well siRNA into rows B-O and columns 3-22 in Falcon 384-well Optiplex screen plates.
2. In each screen plate using the liquid handler, spot in siCON and siPINK1 in wells found in columns 2 and 23. Edge wells will not be used in the screen due to edge effects (Fig 2.2).
3. Seal screen plates with aluminum foil plate sealer and store plates at -20°C.
4. Working OptiMEM will be a 2X solution. OptiMEM (cat 22600-050) is shipped in a set 10 packages. Each package contains OptiMEM powder to make 1 L of 1X OptiMEM. To make 2X OptiMEM, resuspend one package in 500 mL of autoclaved H₂O. Make enough for the complete screen. Supplement with 2.4 g of NaHCO₃. Adjust pH to 7.4. Filter sterilize.
5. Heat inactivate FBS by incubating FBS bottle at 56°C for 30 min.
6. Complete DMEM by adding 50 mL of heat-inactivated FBS and 5 mL of Penicillin-Streptomycin solution to 500 mL of DMEM.

Tip: Whenever possible, make enough solution in one batch to cover the complete screen to ensure consistency in results. All cells must be checked for *Mycoplasma* contamination.

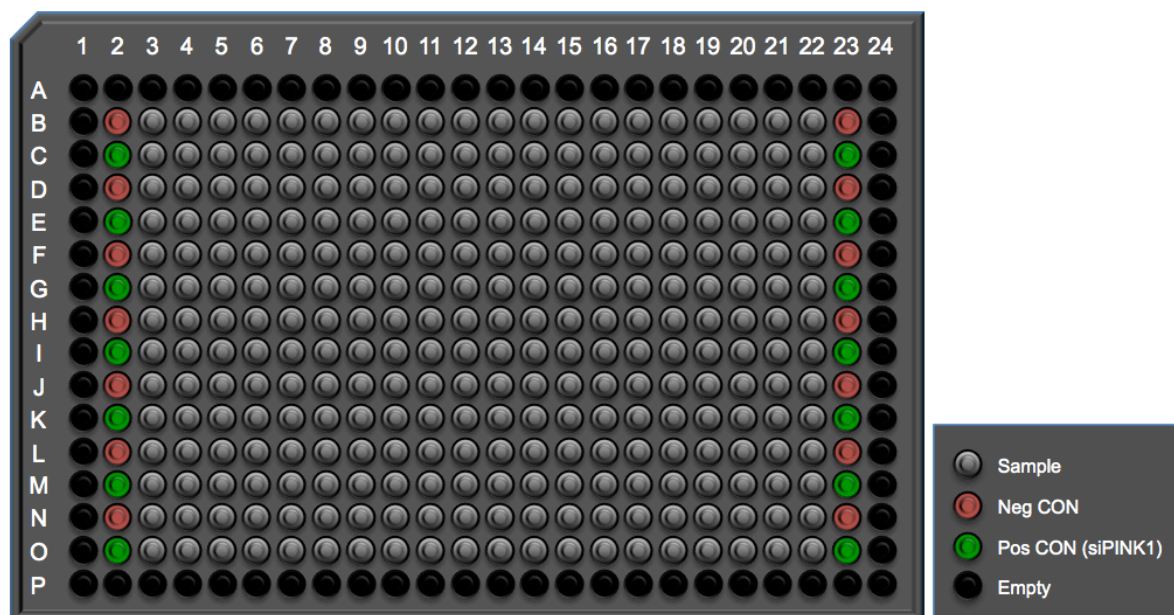


Fig 2.2: Screen map of each screen plate. All edge wells (black wells) contained water only in order to mitigate edge effects. Columns 2 and 23 contained siCON (magenta wells) and siPINK1 (green wells). siRNA from screen libraries were arrayed in the rest of the wells (grey wells).

2.8. Genome Screen Protocol

2.8.1 Duration

Reverse Transfection/Viral Infection	2 h
Incubation	3 Days
High-Throughput Immunofluorescence	2 Days
Automated Microscopy	2 Months

Table 2.4: Duration of genome screen from sample preparation to automated imaging.

2.8.2 Sample Preparation

1. Determine appropriate delivery method of GFP-Parkin into HeLa cells. We find that both AAV and lentivirus worked well.

Tip: During assay development, titrate increasing amounts of virus to obtain sufficient GFP-Parkin fluorescence signal. Prepare enough virus for the entire screen.

2. Thaw screen plates at room temperature for 30 min. Spin plates at 1000 X g for 1 min to collect the siRNA reagent to the bottom of the screen plate.

3. Make a master mix containing 0.025 μ L Lipofectamine RNAiMAX in 5 μ L of 2X OptiMEM. Mix well and incubate for 5 min.

4. Using multidrop, add 5 μ L of RNAiMAX/OptiMEM directly into well containing siRNA.

5. Incubate for 20 min.

6. During the incubation, wash with PBS and trypsinize HeLa cells. Resuspend cells at 450 cells per 30 μ L complete DMEM. Add appropriate titre of AAV or lentivirus-expressing GFP-Parkin.

Tip: Freeze multiple vials of low-passage cells for both assay development and for the screen.

7. After the incubation of the transfection mix, add 30 μ L containing 450 cells into each well. Leave plate at room temperature for 15 min before transferring into 37°C, 5% CO₂ incubator.

8. Incubate for 72 h.

9. Add 10 μ L of 25 μ M CCCP (final: 5 μ M) directly into the well to trigger GFP-Parkin translocation. Return plates into TC incubator and incubate for 1 h.

Tip: During assay development, titrate increasing amounts of CCCP in order to get robust GFP-Parkin recruitment to mitochondria.

10. Prewarm fixation solution consisting of complete medium containing 7.4% formaldehyde and 5 µg/mL Hoechst 33342 (Final: 3.7% formaldehyde and 2 µg/mL Hoechst 33342)

11. Using multidrop, add 50 µL of fixation solution into each well of screen plates.

12. Incubate for 1 h.

Tip: After the multidrop is finished dispensing reagent in the final screen plate, the first plate should be ready for the next step.

13. Using BioTek plate washer, rinse each plate with 0.75% glycine in PBS.

14. Aspirate with plate washer, and dispense 30 µL of 0.1% TX100 in PBS using multidrop.

15. Incubate for 1 h.

16. Using BioTek plate washer, rinse each plate with PBS with final aspirate selected.

17. Dispense 30 µL of 3% BSA in PBS.

18. Incubate for 1 h.

19. Aspirate with plate washer.

20. Dispense 30 µL of 0.1 µg/mL rabbit polyclonal IgG anti-TOMM20 in 3% BSA/PBS.

21. Incubate overnight at 4°C.

22. Using BioTek plate washer, rinse each plate with PBS with final aspirate selected.

23. Dispense 30 μL of 1 $\mu\text{g/mL}$ AlexaFluor® 594 Goat Anti-Rabbit IgG (H+L) in 3% BSA/PBS using multidrop.
24. Incubate for 1 h at room temperature.
25. Using BioTek plate washer, rinse each plate 3 X with PBS.
26. Manually seal plates with transparent plastic seals. Clean plate bottom with 70% EtOH-soaked Kimwipes.
27. Calibrate the position and movement of robotics arm to allow for automated transfer of screen plates from stack to Cellomics-automated microscope.
28. Turn on Cellomics-automated microscope. Apply the following filter set: XF53 (suitable for Hoechst, GFP and AlexaFluor 594). Manually insert a screen plate. Using control wells, adjust detection exposure so that Hoechst, GFP-Parkin, and TOMM20 signal are within the linear range of detection. Please see microscope and algorithm settings for more detail.
29. Stack plates onto plate stacker. The triplicate plates should be imaged one after the other to reduce variability due to signal loss.
30. Perform automated imaging. Each plate should take around 2–3 h depending on the microscope and algorithm settings.

2.8.3 Microscope and Algorithm Settings

Several full test plates with the controls using all the automation equipment should be processed before the actual screen to assure that consistency for the screen can be achieved. From these plates, microscope and algorithm settings can be optimized before the actual

screen is started. The Correlation Coefficient calculations in the colocalization assay protocol of the vHCS Scan software uses the Pearson Correlation Coefficient which will correlate the pixel intensity ratio of each target that overlays each other with the overall average intensity of each target within the region of interest (ROI). This will take into account variability of staining of the TOMM20 and expression of the GFP-Parkin fusion protein from well to well.

Cellomics Arrayscan V^{TI} assay protocol parameters

- Channel 1,2,3 = Hoechst, GFP-Parkin, TOMM20
- 40 X objective, 10 fields, autofocusing each field on Channel 3,
- ROI_A = 40 pixel radius around segmented nucleus,
- Target1 = GFP-Parkin fusion protein
- Target2 = Tomm20 staining of mitochondria
- ROI_A_CorrelationCoefLevelLow = 0.8

1. For nucleus selection, scan random wells of test plates and adjust background correction, object segmentation and object identification cutoff so that all nuclei are properly selected and segmented if in a cluster.

2. Adjust minimum area size of nucleus to remove debris stain in Channel 1 and if desired, set a maximum average intensity cutoff of nucleus to remove cells that are dying or undergoing mitosis.

3. In the GFP-Parkin channel adjust background correction and set object identification to a variable detection method like Isodata Threshold, adjusting cutoff to detect GFP signal and

not background. Adjust a minimum total intensity cutoff so cells not expressing GFP-Parkin are not measured.

4. Use a fixed object identification method to detect mitochondria in Channel 3 adjusting the object cutoff and background correction to achieve the best results (Fig 2.3).

Tip: Adjust the minimum object area of detected mitochondria to a small value to remove background from the mitochondria channel but allowing the object cutoff value to be lowered to detect more mitochondria.

5. Examine the ROI_A_Correlation_Coefficient of cells that have been treated with and without CCCP and the various controls. Determine a cutoff value where lower values would be cells where Parkin would be considered cytoplasmic and are not recruited into the mitochondria. Enter this cutoff value to ROI_A_CorrelationCoefLevelLow in the vHCS Scan software to get percentage of cells with solely cytoplasmic GFP-parkin.

6. Select cell features to store in the output of the data image analysis to minimize the amount of data generated.

2.8.4 Data Analysis

We used cellHTS2 [37], an open source R Bioconductor software package to analyze the data and rank samples according to the value of percentage low ROI A Correlation Coefficient. This analysis can be coupled with pathway analysis using HTSanalyzeR [38], a Bioconductor software package that integrates with CellHTS2 for pathway analysis. CellHTS2 requires five parameter files to function properly along with a separate text file for each plate containing the results data. The files are tab-delimited plain text files.

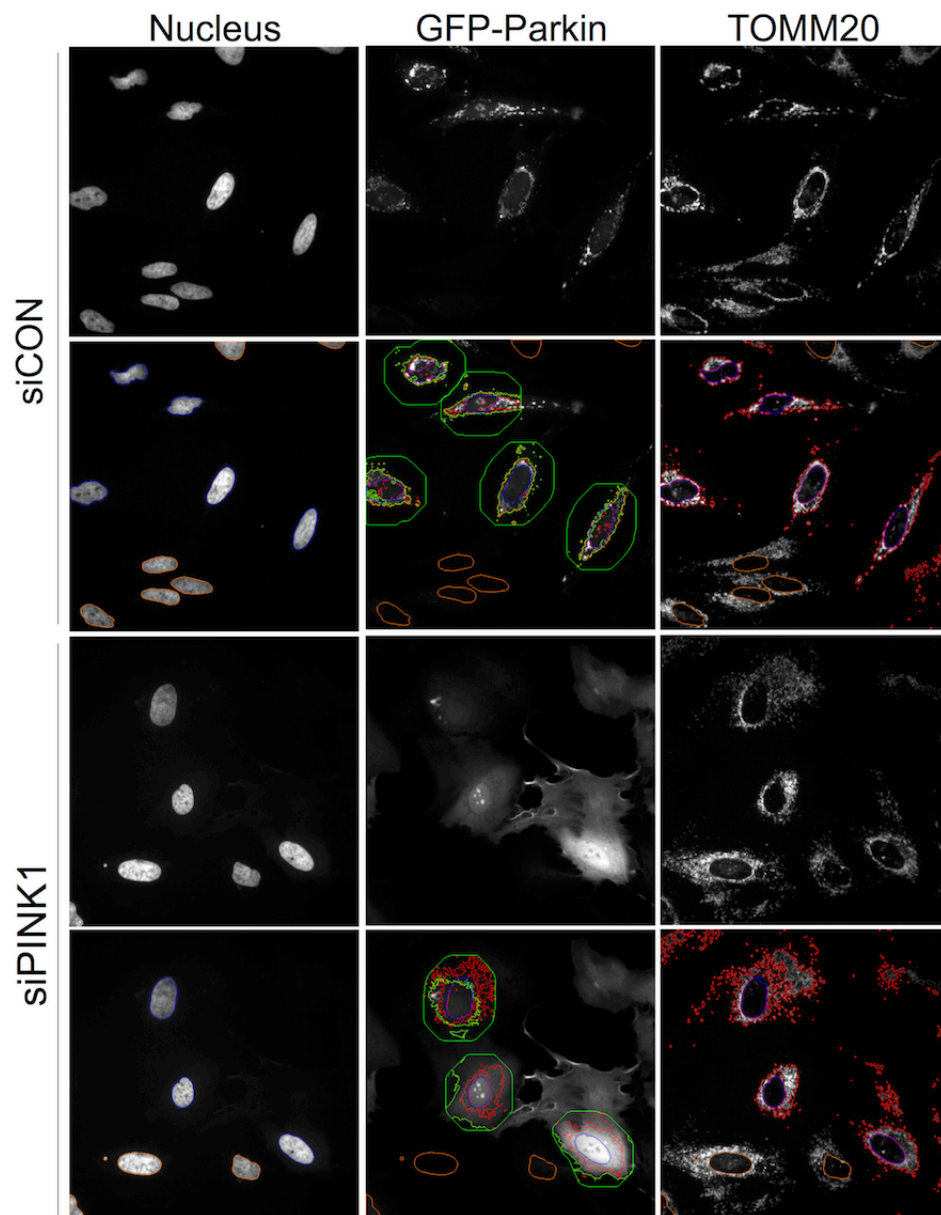


Fig 2.3: Example fluorescence micrographs of siCON and siPINK1 wells from the genome-wide screen. siCON and siPINK1-transfected cells expressing GFP-Parkin were treated with CCCP, fixed, and stained with Hoechst 33342 and TOMM20 antibody. In the second and fourth rows of images, masks were drawn based on the fluorescence signal detected. Red masks were drawn around TOMM20 fluorescence. Green masks were drawn around GFP fluorescence. Nuclei of cells expressing a minimum of GFP-Parkin are circled in blue, those below the minimum are circled in orange. The green mask is shown 40 pixels from the edge of nuclei in the GFP-Parkin channel overlays. The correlation coefficient between GFP-Parkin and mitochondria signal was calculated and a threshold was defined to determine the percentage of cells with low-signal overlap (ie: blocked Parkin translocation).

2.8.4.1 Parameter Files:

1. Description.txt: description of the screen, but file is optional or can be left empty.
2. geneID.txt: Tab delimited list giving Entrez Gene ID and Gene Symbol.

Plate	Well	GeneID	GeneSymbol
1	B01	NA	NA
1	B02	NA	NA
1	B03	3354	HTR1E
1	B04	222611	GPR111

3. Plateconf.txt: plate map file-designating controls

Wells: 384		
Plates: 66		
Plate	Well	Content
*	*	sample
*	B01	empty
*	B02	neg
*	C02	pos
*	D02	other

4. Platelist.txt: List of filenames of the data for each plate.

Filename	Plate	Replicate
h4ds0101D007.txt	1	1
h4ds0101D008.txt	1	2
h4ds0101D009.txt	1	3
h4ds0202D007.txt	2	1
h4ds0202D008.txt	2	2

5. Screenlog.txt: Flag wells to be ignored during analysis. *Note:* Sample column refers to plate replicate number. Comment column can be any word.

Plate	Sample	Well	Flag	Comment
3	1	G10	NA	LowCellCount

3	2	G10	NA	LowCellCount
3	3	G10	NA	LowCellCount
25	2	D13	NA	Contamination

2.8.4.2 Data files

6. h4ds0101D007.txt

h4ds0101D007	A24	
h4ds0101D007	B01	
h4ds0101D007	B02	62.16
h4ds0101D007	B03	88.52
h4ds0101D007	B04	81.48

2.8.4.3 cellHTS2 commands

The R open source statistical package must be installed and within that the modules that are required for cellHTS2 (see documentation at <http://bioconductor.org/packages/release/bioc/html/cellHTS2.html>) and HTSanalyzeR. In the commands following dataPath = directory path where the parameter and data files are kept, dataPathPathway is where the pathway analysis result files will be written to. To set dataPath for the directory you are in (dataPath <- "."). After starting R, the following commands are used:

```

> library("cellHTS2")
Load library files for pathway analysis
> library(org.Hs.eg.db)
> library(GO.db)
> library(HTSanalyzeR)
> library(KEGG.db)
> library(BioNet)
Start cellHTS2 analysis

```

```

› plate<-readPlateList("Platelist.txt", name="controls",
  path=dataPath)
› plate<-configure(plate, descripFile="Description.txt",
  confFile="Plateconf.txt", logFile="Screenlog.txt",
  path=dataPath)
› plate<-annotate(plate, "geneID.txt", path=dataPath)

```

Normalize plate with normalized percent inhibition (NPI) but if plates have uneven reading due to a spatial effect on the plate, redo the analysis, use a normalization method like the B-score method (*Bscore*).

```

› platenor<-normalizePlates(plate, scale="multiplicative",
  method="NPI")
› platesc<-scoreReplicates(platenor, sign="+",
  method="zscore")
› platesc<-summarizeReplicates(platesc, summary="median")
› platesc<-annotate(platesc, "geneID.txt", path=dataPath)
› setSettings(list(plateList=list(reproducibility=list(include=TRUE,
  map=TRUE), intensities=list(include=TRUE, map=TRUE)),
  screenResults=list(htmlMaxItems=100000)))
› out<-writeReport(raw=plate, normalized=platenor,
  scored=platesc, map=FALSE, force=TRUE, outdir=dataPath)

```

Start of pathway analysis

```

› GO_BP<-GOGeneSets(species="Hs", ontologies=c("BP"))
› GO_CC<-GOGeneSets(species="Hs", ontologies=c("CC"))
› GO_MF<-GOGeneSets(species="Hs", ontologies=c("MF"))
› PW_KEGG<-KeggGeneSets(species="Hs")
› ListGSC<-
  list(GO_BP=GO_BP,GO_CC=GO_CC,GO_MF=GO_MF,PW_KEGG=PW_KEGG)
› HTSanalyzeR4cellHTS2(normCellHTSobject=platenor,
  scoreSign = "+", scoreMethod = "zscore",
  summarizeMethod = "median", annotationColumn = "GeneID",
  species = "Hs", initialIDs =
  "Entrez.gene",duplicateRemoverMethod = "max", orderAbsValue =
  FALSE, listOfGeneSetCollections=ListGSC,cutoffHitsEnrichment =
  2, pValueCutoff = 0.05, pAdjustMethod = "BH", nPermutations =
  1000, minGeneSetSize = 20,

  exponent = 1, keggGSCs=c("PW_KEGG"),
  goGSCs=c("GO_BP","GO_CC","GO_MF"),nwStatsControls = "neg",
  nwStatsAlternative = "two.sided", nwStatsTests = "T-
  test",nwStatsColumns = c("t.test.pvalues.two.samples",
  "t.test.pvalues.one.sample"),nwAnalysisFdr = 0.001,

```

```
nwAnalysisGenetic = FALSE, interactionMatrix =
NULL,nwAnalysisOrder = 1, ntop = NULL,allSig = TRUE, reportDir
= dataPathPathway, verbose = TRUE)
```

When the analysis is finished, the results can be seen by opening the index.html file in a browser. A multi-tab webpage will show a large variety of data including correlation between replicates, a number of heatmaps of the plates, and a ranked list of the genes relative to the controls using a robust z score.

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Chapter 3

Essential role of TID1 in Maintaining Mitochondrial Membrane Potential Homogeneity and Mitochondrial DNA Integrity

3.1 Preface

This chapter contains a published paper on *TIDI*, a gene that we have discovered through a genome screen for genes that regulate mitochondrial dynamics. This work was published in Ng et al, Molecular and Cellular Biology, 2014. We have characterized the role of *TIDI* in mitochondria and found that it prevents aggregation of complex I subunits and prevents the loss of mitochondrial DNA. I have spent the bulk of my studies (>60%) of my Ph.D work on this project. In this chapter, the term $\Delta\psi$ is used to refer to $\Delta\psi_m$, mitochondrial membrane potential, and should be distinguished from $\Delta\psi_o$, the plasma membrane potential.

Author list

Andy Cheuk-Him Ng, Stephen D. Baird, Robert A. Screaton

Author contribution

AC-HN designed and performed all the experiments listed in this manuscript. SDB was instrumental in the genome screen and in the designing of algorithm used to quantify fluorescence signal in confocal microscopy. AC-HN wrote the first draft of this manuscript. RAS helped with experimental design, revised the manuscript and supervised the study.

3.2 Abstract

The tumorous imaginal disc 1 (TID1) protein localizes mainly to the mitochondrial compartment, wherein its function remains largely unknown. Here we report that TID1 regulates the steady-state homogeneity of the mitochondrial membrane potential ($\Delta\psi$) and maintains the integrity of mitochondrial DNA (mtDNA). Silencing TID1 with RNA interference leads to changes in the distribution of $\Delta\psi$ along the mitochondrial network, characterized by an increase in $\Delta\psi$ in focal regions. This effect can be rescued by ectopic expression of a TID1 construct with an intact J domain. Chronic treatment with a low dose of oligomycin, an inhibitor of F_1F_0 ATP synthase, decreases the cellular ATP content and phenocopies TID1 loss of function, indicating a connection between the disruption of mitochondrial bioenergetics and hyperpolarization. Prolonged silencing of TID1 or low-dose oligomycin treatment leads to loss of mtDNA and consequent inhibition of oxygen consumption. Biochemical and colocalization data indicate that complex I aggregation underlies the focal accumulation of $\Delta\psi$ in TID1-silenced cells. Given that TID1 is proposed to function as a cochaperone, these data show that TID1 prevents complex I aggregation and support the existence of a TID1-mediated stress response to ATP synthase inhibition.

3.3 Introduction

In addition to their role in generating ATP from nutrients, mitochondria have been implicated in a wide range of biological processes, such as cell death, cell cycle progression, nutrient flux, and development. Central to the health of mitochondria is the maintenance of an electrochemical potential ($\Delta\psi$) generated by megadalton respiratory complexes collectively known as the electron transport chain (ETC) [1]. Electrons are funneled through the ETC, shuttling protons from the matrix to the intermembrane space. The electromotive force generated by the charge and pH difference propels protons back into the matrix through the F_1F_0 ATP synthase. An intact $\Delta\psi$ is a requirement for cytosolic precursor proteins to translocate to the mitochondrial matrix. Furthermore, uptake of calcium and metabolites is also dependent on $\Delta\psi$ [1, 2]. The loss of mitochondrial $\Delta\psi$ is a critical signal for the initiation of macroautophagy of mitochondria, or mitophagy, a crucial mitochondrial quality control system to remove damaged organelles [3-6]. In the context of programmed cell death, the dissipation of $\Delta\psi$ appears to be an early event preceding the release of cytochrome C, leading to the formation of the apoptosome and downstream effectors [7, 8].

Mitochondrial disorders are the most frequent cause of inborn defects in metabolism, with an estimated incidence of 1 in 10,000 live births [9, 10]. Isolated NADH dehydrogenase (complex I) deficiency is the most frequently diagnosed form (33%) of mitochondrial disorders [11]. Clinical symptoms of complex I deficiency appear at birth or in early childhood, with energetically-demanding tissues being the most affected [12]. Complex I deficiency is associated with childhood diseases such as Leigh syndrome, a fatal neurodegenerative disorder, and syndromes of fatal infantile lactic acidosis (FILA) [13].

Aging-related disorders such as Parkinson's disease (PD) [14] and cancer [15] are associated with complex I defects as well. Several lines of evidence suggest that promoting complex I activity, via ectopic introduction of *Saccharomyces cerevisiae* NDI1 protein, prevents neuronal loss in PD [16] and Leber hereditary optic neuropathy (LHON) [17], and increases longevity [18]. However, the 950 kDa NADH dehydrogenase enzyme is the largest but least understood of the respiratory complexes [19]. Interestingly, the loss of function of two PD genes *HtrA2* and *PINK1* promotes the accumulation of misfolded mitochondrial proteins, including subunits of the NADH dehydrogenase enzyme [20, 21].

Tumorous imaginal disc 1 (TID1) is a member of DnaJ/Hsp40 family of molecular chaperones. Proteins of the Hsp40 family share a DnaJ domain that serves to stimulate the ATPase activity of Hsp70 during protein refolding and transport. In mammalian cells, HSPA9/mortalin is the only mitochondrial Hsp70 family member and was proposed to function with TID1 as its co-chaperone [22]. Two TID1 mRNA splice forms that encode TID1 long and short isoforms which act as DnaJ cochaperones in the cytosol exist [23-26], and are processed into mature forms following import into the mitochondrial matrix. Tid1-knockout mice die between embryonic day 4.5 (e4.5) to e7.5, demonstrating a requirement for Tid1 for normal development [27]. Furthermore, mice with conditional loss of Tid1 in the heart died before age 10 weeks due to dilated cardiomyopathy [28].

To date, a mitochondrial substrate for TID1-mortalin has not been identified; thus it remains unclear if TID1 functions as a cochaperone for mortalin in mitochondria [29]. Here, we show that under basal conditions, TID1 is associated with mitochondrial DNA (mtDNA) and

complex I. Following chronic inhibition of ATP synthase, the TID1 protein accumulates and shifts from a detergent-soluble, mtDNA-associated fraction into a detergent-insoluble fraction where it preferentially associates with complex I. In TID1-knockdown cells, complex I colocalizes with hyperpolarized regions of the mitochondrial network and accumulates in a detergent-insoluble fraction, which can be reversed by overexpression of TID1. We propose a model whereby ATP synthesis inhibition induces a stress response involving TID1-mediated refolding of complex I subunits.

3.4 Materials and Methods

3.4.1 Tissue culture

U2OS cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal calf serum. Parental 143B and 143B Rho0 cells were additionally supplemented with pyruvate and uridine [30].

3.4.2 Reagents

Oligomycin, *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP), and antimycin were from Seahorse Biosciences. *N*-Acetylcysteine (NAC), carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), and rotenone were from Sigma. Glucose oxidase was from Roche.

3.4.3 Antibodies

Antibodies to TID1 (RS-11), mortalin, and TOMM20 (FL-145) were from Santa Cruz. Antibodies to NDUFS3 (3F9DD2) and MT-COII (EPR3314) were from Abcam. A monoclonal antibody cocktail consisting of NDUFA9, SDHA, UQCRC2, and ATP5A antibodies was from Invitrogen (catalog no. 457999). Antibody to DNA was from Progen.

MitoTracker Red (MTR) CM-H2 XROS (M7513) was from Molecular Probes. Tetramethylrhodamine ethyl ester (TMRE) was from Invitrogen.

3.4.4 RNA interference (RNAi)

The target sequences of the small interfering RNA (siRNA) and short hairpin RNA (shRNA) used in Figure S3.1 in the supplemental material are as follows:

si1 5'-GCGCAGACATCCACTCCGA-3'; si2 5'-GAAGCAAGGCTAGGCGTGA-3'; si3 5'-TATTAGGAGTGCCTCGAAA-3'; si4 5'-GGATTAACAGCTACGGCTA-3'; shRNA 5'- TCCGACCTCTTTATTTCTATA-3'.

si1 and shRNA were used in the study described in this report. shRNA was delivered via lentivirus and permitted long-term stable knockdown studies, such as in those whose results are presented in Figure 3.4D.

3.4.5 Lentivirus

cDNAs encoding TID1 short and long isoforms were obtained from Addgene plasmids 13709, 13707, respectively, as described in reference [23]. Site-directed mutagenesis was performed to generate the H121Q mutant. *attB1*-flanked TID1 cDNAs were amplified and gateway recombined into pDONR-221. pDONR-TID1 was recombined with pLenti6-V5 DEST to generate pLenti6-TID1 lentiviral expression plasmid. To generate lentiviral shRNA constructs, oligonucleotides encoding short hairpins targeting human TID1 and mouse Tid1 transcripts were ligated into pLKO.1 via AgeI and EcoRI sites. Control shRNA was from Sigma. Lentivirus was prepared as described previously [31].

3.4.6 QPCR

Total genomic DNA was collected and isolated from dimethyl sulfoxide (DMSO)- or oligomycin-treated U2OS cells using Flexigene DNA kit (Qiagen). Genomic DNA (0.5 ng) was used as template for quantitative PCR (QPCR). The relative standard curve method was

used to determine relative fold change from the value for the control. Primer sets described previously [32] were taken to amplify mtDNA. The following primer set targeting the mtDNA 12S RNA gene (MT-RNR1) were used: forward primer 5'-GCTCGCCAGAACACTACGAG-3' and reverse primer 5'-CAGGGTTTGCTGAAGATGGCG-3'. To amplify nuclear DNA, the following primer set targeting EEF1A1 was used: 5'-GGATTGCCACACGGCTCACATT-3' and 5'-GGTGGATAGTCTGAGAAGCTCTC-3'.

3.4.7 Bioenergetics Assays

U2OS cells were treated with lentivirus expressing either CON or TID1 shRNA. For measurement of the effect of mtDNA loss on bioenergetics, measurements were done 10 days after lentivirus introduction. For more acute effect of TID1 knockdown, measurements were done 4 days after lentivirus infection. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a measure of glycolytic flux, were determined using Seahorse XF24 assay (Seahorse Bioscience). Briefly, 24 h before the assay, 50,000 infected cells were plated onto Seahorse 24-well culture plates. On the day of measurement, XF24 assay medium supplemented with 25 mM glucose was applied to cells and equilibrated at 37°C under ambient O₂ and CO₂ conditions for 30 min. OCR and ECAR were measured with an XF24 analyzer. The following mitochondrial inhibitors at the indicated final concentrations were injected via injection ports on the Seahorse cartridge plate: 0.5 μM oligomycin, 1 μM FCCP, and 0.5 μM antimycin.

3.4.8 Immunofluorescence and confocal microscopy

Cells seeded in 384-well imaging plates were fixed using formaldehyde or methanol (for TID1 staining) and imaged with a X63 water objective (numerical aperture = 1.2) on an Olympus Fluoview FV1000 confocal laser scanning microscope. For quantitation of the

percentage of cells displaying heterogeneous mitochondrial hyperpolarization (hot spots), stained cells in imaging plates were imaged at X20 objective with the Opera high-content screening system (PerkinElmer). At least 20 fields per well containing at least 1,000 cells were scored. For rescue experiments, only cells expressing exogenous TID1, as determined by TID1 fluorescence positivity, were scored. Statistical analyses were performed using Student's *t* test. A *P*-value <0.05 was considered significant, and where appropriate, *P* values are indicated in all figure legends. All error bars are presented as standard errors of the means.

For depolarization experiments, cells were loaded with 40 nM TMRE and a small region of interest (ROI) was irradiated with a 405 nm laser at 50% power. Recovery of TMRE along the entire length of the mitochondrion was monitored every 2 s. Line scan analyses were performed using Fluoview software by drawing a line along a straight mitochondrion (marked by TOMM20 fluorescence signal). For CCCP washout experiments, cells were first treated with RNAi for 5 days, as indicated below. DMEM with 10 μ M CCCP was added, and the cells were incubated for 1 h to depolarize mitochondria. For washout phase, medium was removed and replaced with medium containing either DMSO, 2 μ M antimycin, or 100 nM rotenone, but no CCCP. MTR (300 nM) was then added for 30 min. Live image acquisition and quantitation of mitochondrial hyperpolarization were performed using an Opera confocal microscope and Columbus software.

3.4.9 Electron microscopy

U2OS cells ($n = 125,000$) were treated with CON or TID1 RNAi for 5 days in a 6-well dish. Cells were washed in phosphate-buffered saline (PBS), trypsinized, and resuspended in 2% glutaraldehyde-PBS for embedding as described previously [33].

3.4.10 Mitochondrial Fractionation

Detergent solubility experiments were performed as follows: U2OS cells were treated as indicated above. Cells were harvested by trypsinization and washed with ice-cold PBS. Digitonin (1%) in PBS supplemented with protease inhibitor cocktail (Pierce) was added to the cells for 30 min on ice. Extracts were centrifuged at 20,000 X g at 4°C. Supernatant was separated from insoluble material (pellet). SDS sample buffer was added to both pellet and supernatant for SDS-PAGE/Western blotting. For analysis of the TID1 association with mtDNA, a brief experimental schematic is shown in Figure 3.5E. Briefly, mitochondria were isolated by differential centrifugation (heavy membrane fraction, 7000 g), and lysed with 0.5% NP-40 in mitochondrial isolation buffer (200 mM sucrose, 10 mM Tris-MOPS [morpholinepropanesulfonic acid], 1 mM EGTA-Tris, pH 7.4) with or without 10 mM spermidine. Centrifugation was performed at 20,000 X g for 30 min at 4°C to obtain pellet (P0) and soluble (S0) fractions prior to solubilization in 1X SDS sample buffer for Western blotting. For DNase I treatment, the P0 fraction was resuspended in reaction buffer (40 mM Tris HCl, pH 7.5, 8 mM MgCl₂, 5 mM dithiothreitol, 0.5% NP-40) and incubated for 45 min at 4°C with 15 U of DNase I. The reaction mixture was centrifuged at 20,000 X g for 30 min at 4°C to generate P1 and S1 fractions for SDS-PAGE, as described above.

3.4.11 Immunoprecipitation

Cells from two 15-cm² plates of confluent U2OS cells were harvested by trypsinization and washed with ice-cold PBS following the indicated treatments. Cells were lysed in coimmunoprecipitation (co-IP) lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM EDTA, 5% glycerol) for 30 min at 4°C and clarified by centrifugation at 20,000 X g for 30 min at 4°C. Supernatant was precleared with protein A/G beads (Santa Cruz) and 0.5 µg of polyclonal TID1 antibody (PA5-27226; Pierce) or rabbit IgG and protein A/G was

added to precipitate the TID1. Beads were washed with co-IP lysis buffer containing 500 mM NaCl and processed for SDS-PAGE and Western blotting.

3.5 Results

3.5.1 TID1 is required to maintain steady-state distribution of $\Delta\Psi$

During the course of a genome-wide RNAi screen to identify genes that regulate mitochondrial $\Delta\Psi$, we observed that knockdown of TID1 produced a unique, highly intense, punctate pattern of MitoTracker Red (MTR) staining, which we termed ‘hot spots’ (Fig 3.1a). Hot spots were consistently beyond saturation, compared to MTR signal in control cells and were observed using multiple RNAi duplexes targeting different regions of both long and short transcripts of TID1 (Fig S3.1). With higher magnification, hot spots that were not the result of general condensation of the network at those sites, as indicated by TOMM20 staining, could be seen in distinct subregions of the mitochondrial network (Fig 3.1A line scans).

As MTR fluorescence in mitochondria can be sensitive to the oxidation state of the probe, we labeled live cells with the $\Delta\Psi$ -sensitive redox-insensitive probe tetramethylrhodamine ethyl ester (TMRE) at nonquenching concentration (< 50 nM), where fluorescence intensity of TMRE correlates linearly with $\Delta\Psi$ [34]. TID1 silencing resulted in an increase in TMRE fluorescence in focal regions of the mitochondrial network (Fig 3.1B), confirming the MTR data. Furthermore, as expected, acute treatment with the protonophore CCCP and the ATP synthase inhibitor oligomycin resulted in linear loss and increase, respectively, in both MTR and TMRE fluorescence signals (Fig S3.2). Thus, we conclude that TID1 silencing specifically affects $\Delta\Psi$. We elected to use MTR for subsequent measurements of $\Delta\Psi$ as the

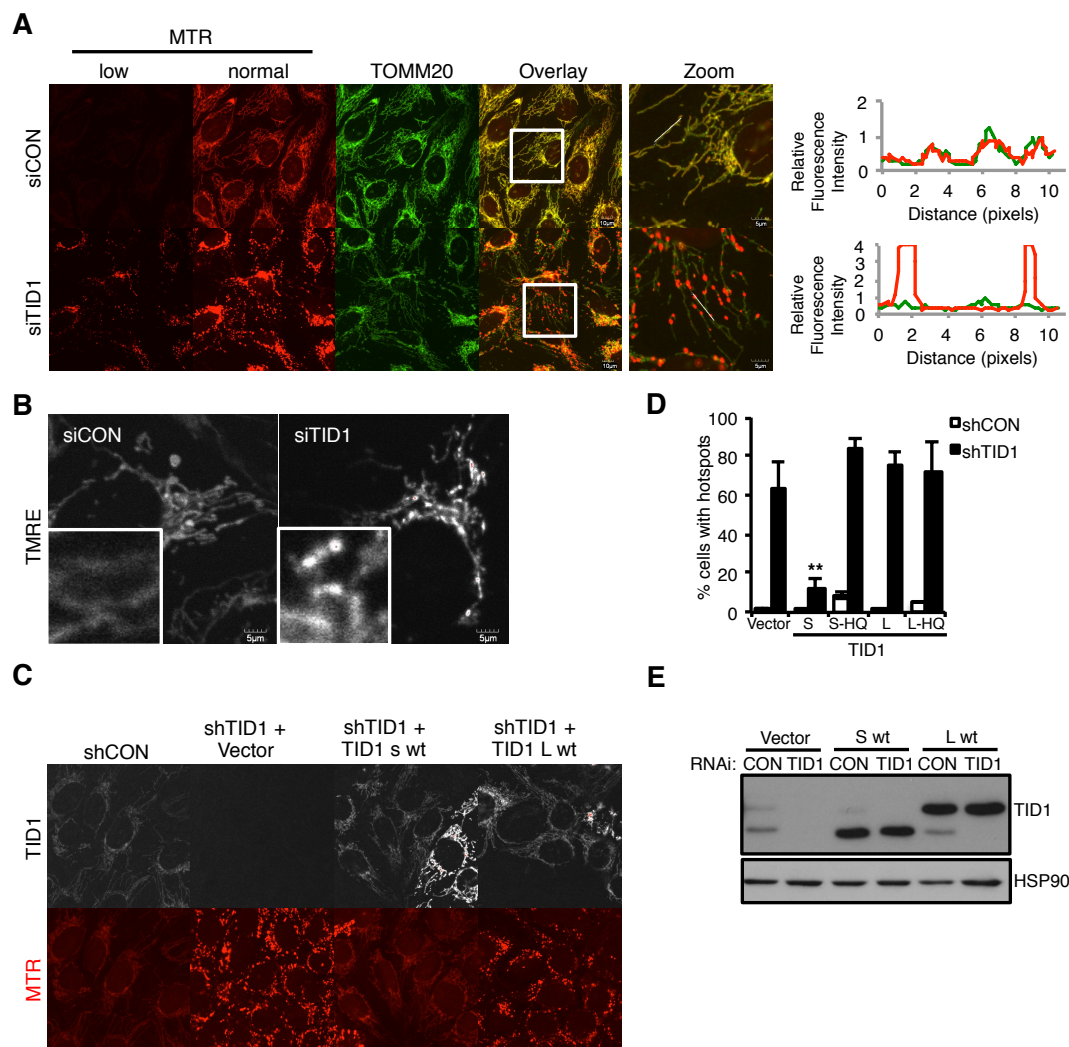
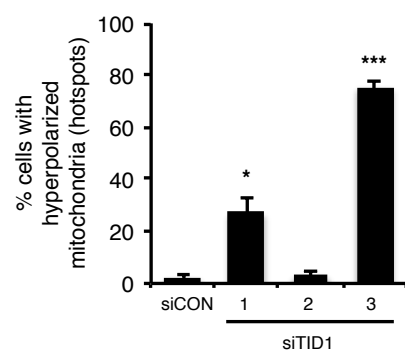


Fig 3.1: TID1 is required to maintain a homogeneous distribution of $\Delta\Psi$. (A)

Fluorescence micrographs of CON- or TID1-silenced cells stained for MTR (red) and TOMM20 (green). Boxed areas in the Overlay column are magnified in the Zoom column. Detection of the MTR channel set to low or normal is shown. Line graphs showing the pixel intensity as a function of distance are shown at the right. Red line, MTR intensity; green line, TOMM20 stain intensity. (B) Fluorescence micrographs showing CON- or TID1-silenced cells loaded with 40 nM TMRE. (C) Fluorescence micrographs showing the effect of ectopic expression of RNAi-resistant TID1 constructs. TID1 is shown in grey scale. MTR is shown in red. (D) Bar plot showing quantitation of the rescue experiment from panel C. **, $P < 0.05$ compared to shRNA against TID1 plus the vector. (E) Western blot showing knockdown and reconstitution of TID1 proteins in panels A to C. siCON, siRNA against CON; siTID1, siRNA against TID1; shCON, shRNA against CON; shTID1, shRNA against TID1; S-HQ, TID1-S H121Q; L-HQ, TID1-L H121Q; wt, wild type.

A



B

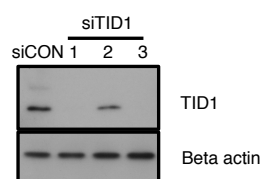


Fig S3.1: Multiple RNAi reagents targeting TID1 result in hot spot formation. (A) Bar plots showing % cells with hotspots in cells treated with siCON or siTID1 targeting three separate regions of TID1 transcript. Data are manually scored, n>200 cells. *p<0.05 and ***p<0.005 values compared to CON siRNA. (B) Western blot showing knockdown with reagents from S3.1A.

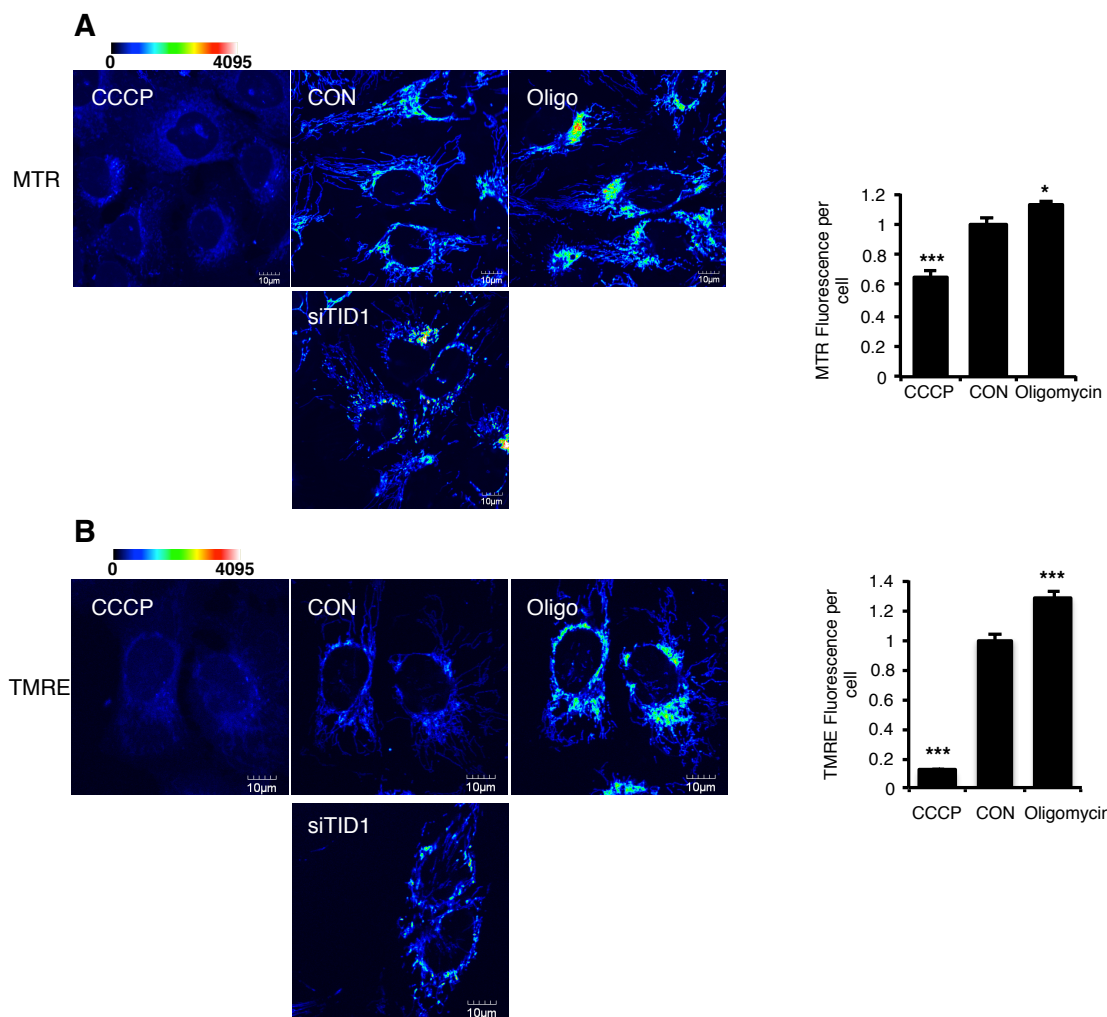


Fig S3.2: Mitochondrial hotspots in TID1 knockdown cells and in cells treated with CCCP and oligomycin. Fluorescence micrographs showing cells treated for 10 minutes with 20 μ M CCCP, DMSO (CON) or 5 μ M oligomycin and subsequently loaded with 350 nM MTR (A) or 40 nM TMRE (B). Images were pseudocoloured to display relative intensity changes. Bar plots on the right show mean fluorescence per cell with the various treatments.

covalent conjugation of MTR probe to mitochondrial proteins allows postfixation immunocytochemical analyses and provided higher hot spot intensity.

To confirm the specificity of TID1 silencing, we generated siRNA-resistant TID1 short (TID1-S) and long (TID1-L) expression constructs using site-directed mutagenesis and expressed these in cells in which TID1 had been silenced. Following reintroduction of TID1-S alone (Fig 3.1C-E) or TID1-L and TID1-S together (Fig S3.3), but not TID1-L alone, we observed restoration of homogenous $\Delta\Psi$, indicating that TID1-S is specifically required for maintaining homogenous $\Delta\Psi$. The J domain of TID1 contains a 3-amino-acid HPD motif required to promote Hsp70 chaperone activity. Introduction of TID1-S J-domain mutant (TID1 H121Q) was unable to rescue the TID1 loss-of-function phenotype (Fig 3.1D), indicating that the J-domain activity of TID1 is required to maintain homogenous $\Delta\Psi$. We noticed that when expressed at higher amounts, the TID1 J-domain mutant also triggered hot spot formation in a manner indistinguishable from silencing of TID1 (Fig S3.4), suggesting that the H121Q mutant can act as a dominant negative.

As TID1 is anticipated to function as a chaperone pair with mitochondrial Hsp70, we tested whether knockdown of the Hsp70 protein mortalin would also result in hot spots. However, silencing of mortalin resulted in dissipation of $\Delta\Psi$ (Fig S3.5), consistent with the idea that mortalin has TID1-independent functions, such as protein import, protein refolding, and mitochondrial biogenesis [35-37]. Taken together, these data show that TID1 regulates the distribution of mitochondrial membrane potential.

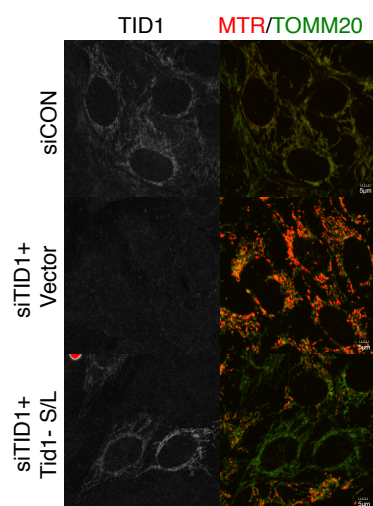
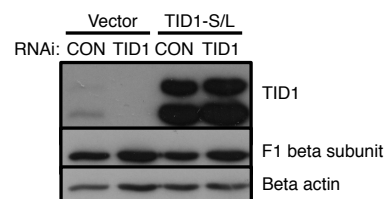
A**B**

Fig S3.3: Reintroduction of TID1- S and L isoforms rescues TID1 knockdown-induced hot spot formation. (A) Fluorescence micrographs showing siCON and siTID1-treated cells stained for TID1-S and TID-L together (gray), MTR and TOMM20. MTR (red) and TOMM20 (green) were shown as merged images. (B) Western blot analysis of rescue experiment.

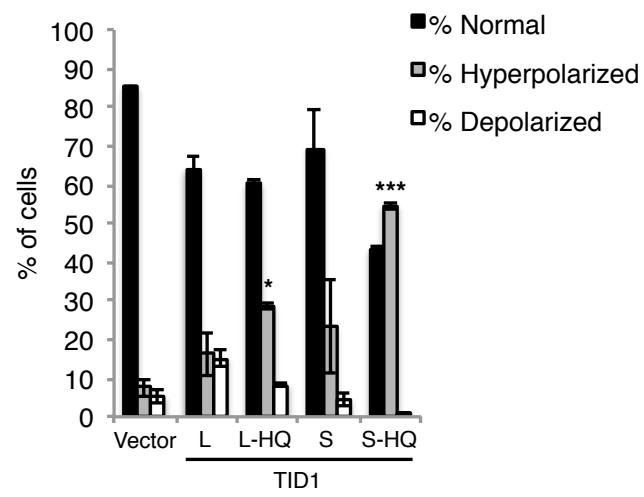


Fig S3.4: TID1-S H121Q is a dominant interfering mutation. Bar plot showing % cells with normal, hyperpolarized, or depolarized mitochondria. Cells treated with a high dose of lentivirus expressing vector control or various TID1 constructs * $p < 0.05$, *** $p < 0.005$ compared to vector % hyperpolarized mitochondria.

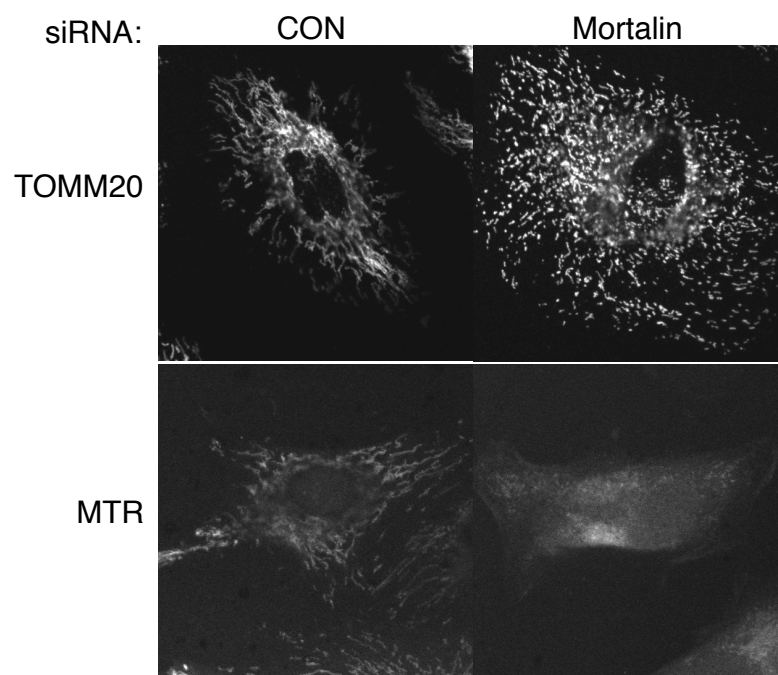


Fig S3.5: Mortalin knockdown leads to mitochondrial membrane depolarization.

Representative fluorescence micrographs showing siCON or siMortalin-treated cells stained with TOMM20 or MTR.

3.5.2 Chronic inhibition of ATP synthase generates hot spots

We next screened a panel of small molecules that target mitochondria to see if we could trigger the formation of $\Delta\psi$ hot spots. Following a 2-day treatment of U2OS cells with inhibitors of the electron transport chain/oxidative phosphorylation and protonophores, as well as reactive oxygen species agonists and antagonists (Fig S3.6), only oligomycin treatment at 20 nM led to hot spots across the mitochondrial network (Fig 3.2A-B), in a pattern indistinguishable from that observed in TID1-knockdown cells. Oligomycin binds to the oligomer of a- and c-ring subunits of the F_o portion of ATP synthase and inhibits proton translocation activity. Short-term treatment (< 1 h) with 1 μ M oligomycin, which achieves acute inhibition of the synthase, resulted in a mild increase in $\Delta\psi$ uniformly over the entire mitochondrial network (data not shown). Expression of TID1-S, but not that of TID1-L wild type or TID1-L H121Q, inhibited oligomycin-induced hot spot formation by $\sim 70\%$, raising the possibility that oligomycin induces hot spot through functional inhibition of TID1 (Fig 3.2B).

3.5.3 A functional respiratory chain is required for hot spots induced by depletion of TID1

$\Delta\Psi$ is established by the opposing activities of respiratory complexes of the electron transport chain and the reentry of protons into the matrix via ATP synthase or a leak. To test the possibility that TID1 silencing induces a change in the submitochondrial localization of respiratory complex proteins, we expressed enhanced green fluorescent protein (eGFP)-tagged NDUFS3 to endogenous levels in order to track complex I in live control and TID1-knockdown cells (Fig 3.3A; see Fig S3.7-3.8). NDUFS3-eGFP did not display the same localization pattern as PRDX3, indicating that ectopic NDUFS3 is not mistargeted to the

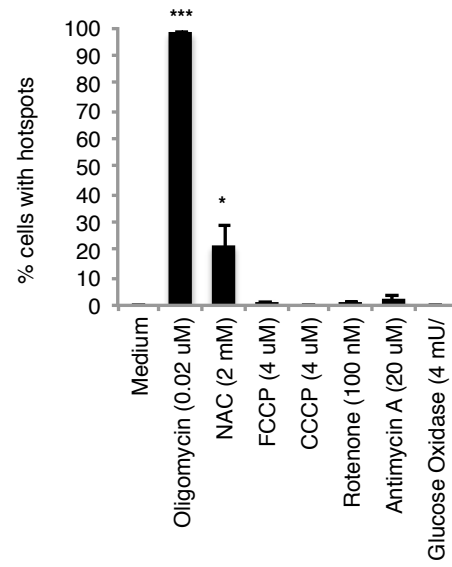


Fig S3.6: U2OS were treated for 2 days with various mitochondrial toxins and stained with MTR for their ability to induce hotspot formation. *** $p < 0.005$ and * $p < 0.05$ compared to DMSO control. $n > 400$ cells per condition were scored.

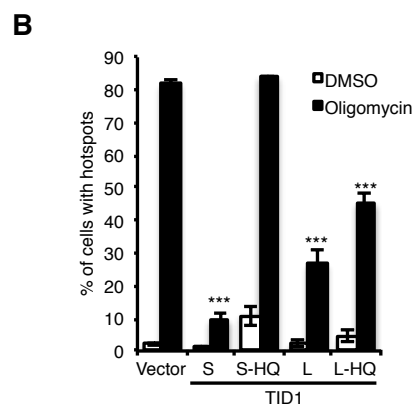
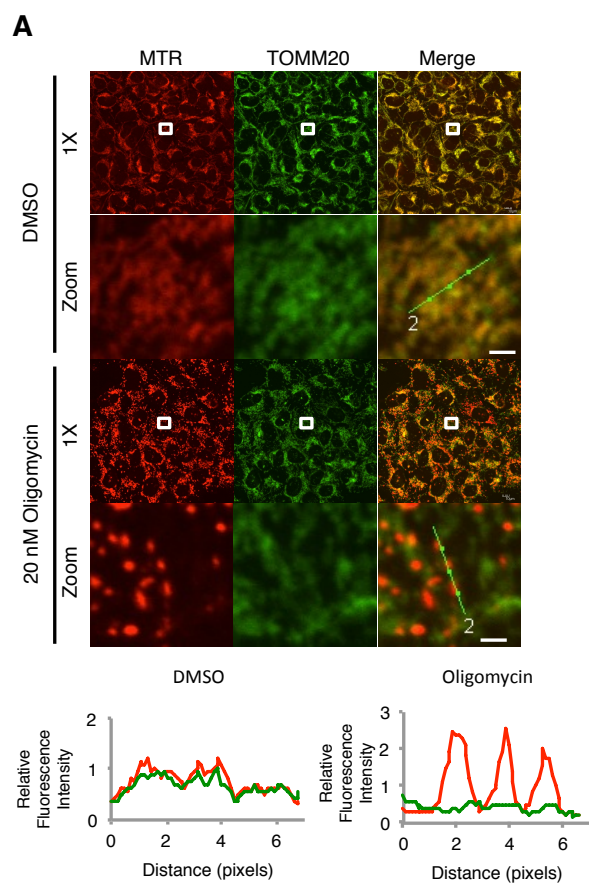


Fig 3.2: Chronic inhibition of ATP synthase generates hot spots. (A) Fluorescence micrographs showing representative images of DMSO- or oligomycin- treated cells. MTR (red), TOMM20 (green), and merged images are shown. Line scan analyses are shown at the bottom of panel A. Green line, TOMM20 staining; red line, MTR staining intensities. (B) Bar plots showing rescue of oligomycin-induced heterogeneous hyperpolarization with TID1 constructs. ***, $P < 0.005$ compared to oligomycin plus the vector. More than 1,000 cells per condition were scored.

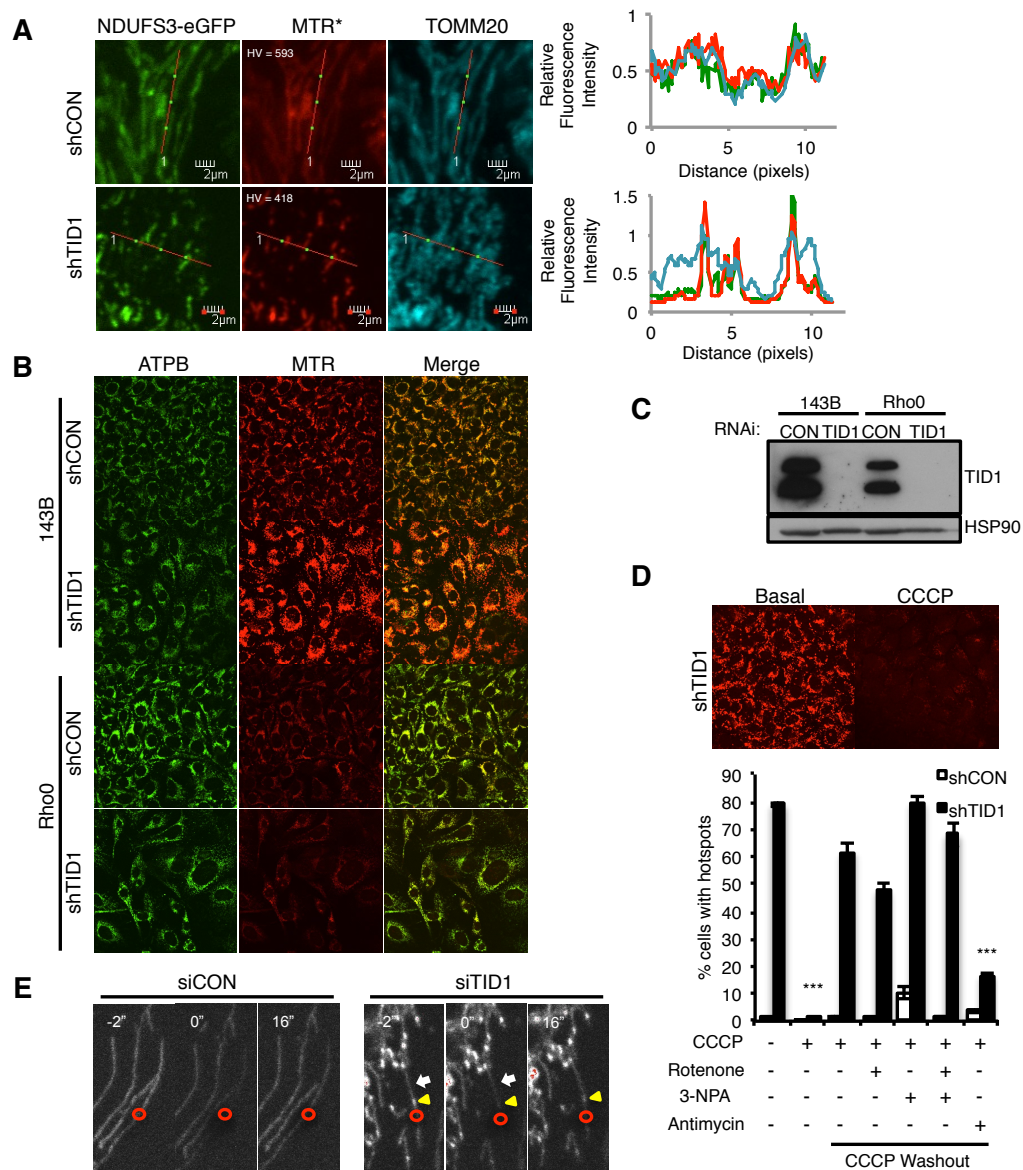


Fig 3.3: A functional respiratory chain is required for hot spots induced by depletion of TID1. (A) Fluorescence micrographs of U2OS cells stably expressing NDUFS3-eGFP treated with shRNA against CON or shRNA against TID1 and subsequently stained with MTR and TOMM20. Line scan analyses are shown at the right. Red line, MTR; cyan line, TOMM20; green line, NDUFS3-GFP staining intensity. MTR*, the sensitivity of MTR detection in fields treated with shRNA against TID1 was decreased and adjusted to a linear range of detection for line scan assessments. The high voltage (HV) of the detection is shown. (B) Fluorescence micrographs of parental 143B and 143B Rho0 cells treated with shRNA against CON or shRNA against TID1 and stained with MTR. ATP synthase subunit beta (ATPB; GenBank name ATP5B) is used as a mitochondrial marker. (C) Western blot from panel B. (D) Fluorescence micrographs showing representative images of cells treated with shRNA against TID1 under basal conditions or conditions with CCCP (10 μ M, 1 h). Bar plots quantify the percentage of cells displaying hot spots when various respiratory chain inhibitors are applied during the CCCP washout phase. 3-NPA, 3-nitropropionic acid. ***, $P < 0.005$ compared to the results for shRNA against TID1 without treatment. (E) Fluorescence micrographs of U2OS cells treated with shRNA against CON or shRNA against TID1 and stained with 40 nM TMRE. The site of irradiation was directed at the region marked by the circles. Fluorescence micrographs of time frames 2 s before irradiation (-2"), during irradiation (0 s [0"]), and 16 s after irradiation (16") are shown. Arrows show a region of the mitochondrion being depolarized when an adjacent region was irradiated; arrowheads point to a hot spot. ***, $P < 0.005$.

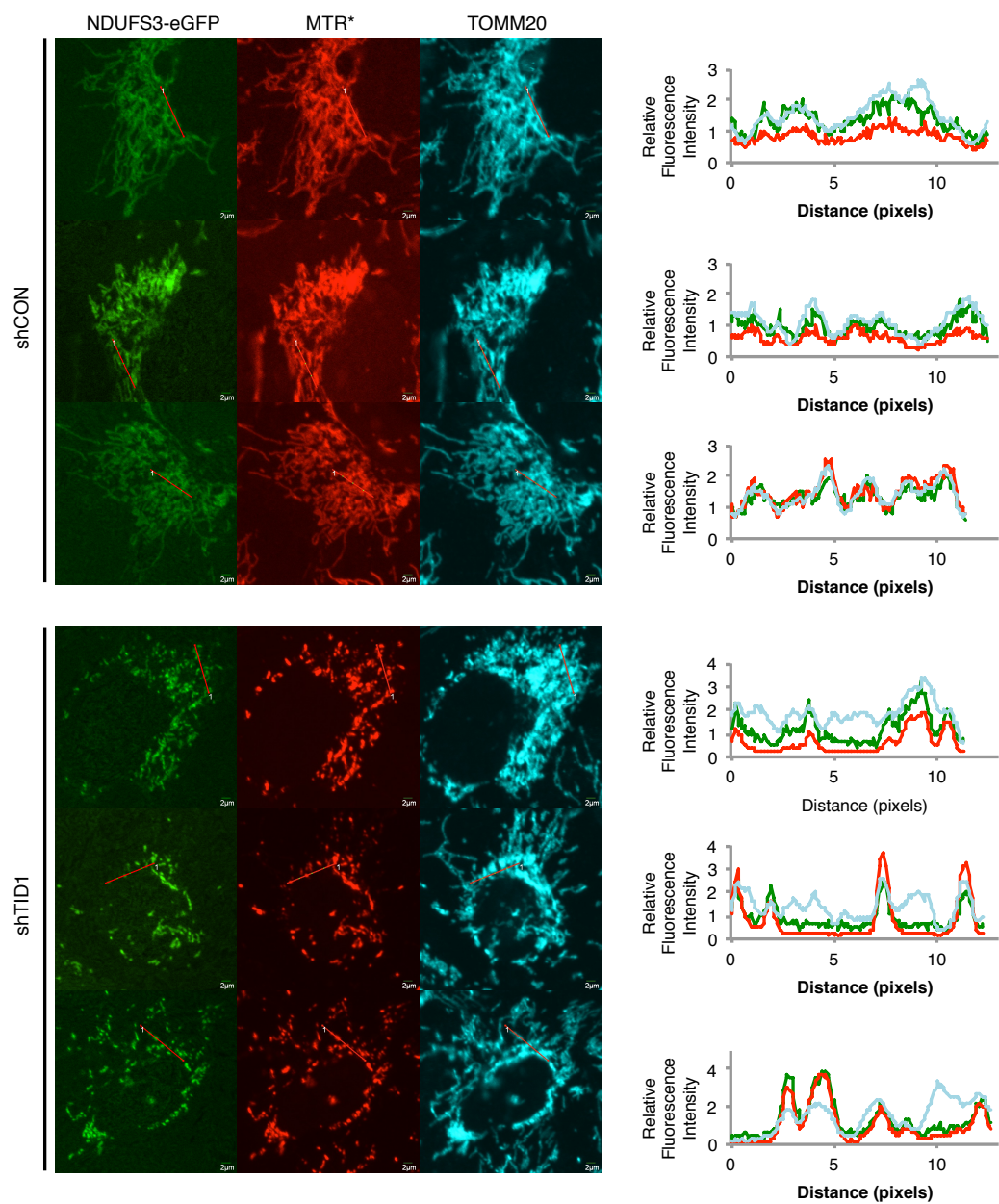
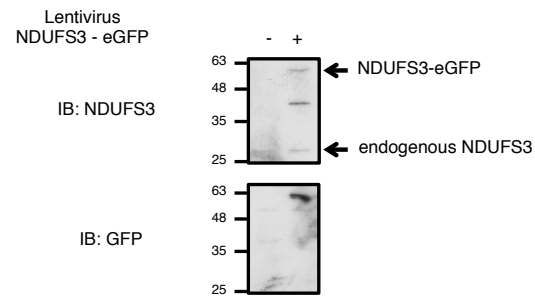


Fig S3.7: NDUFS3, a complex I subunit, localizes to hot spots. Additional representative images (n = 3 cells per condition) from Fig 3.3A are shown. Line scan analyses are shown in right. MTR signal detection is decreased in shTID1 panels to allow for colocalization studies.

A



B

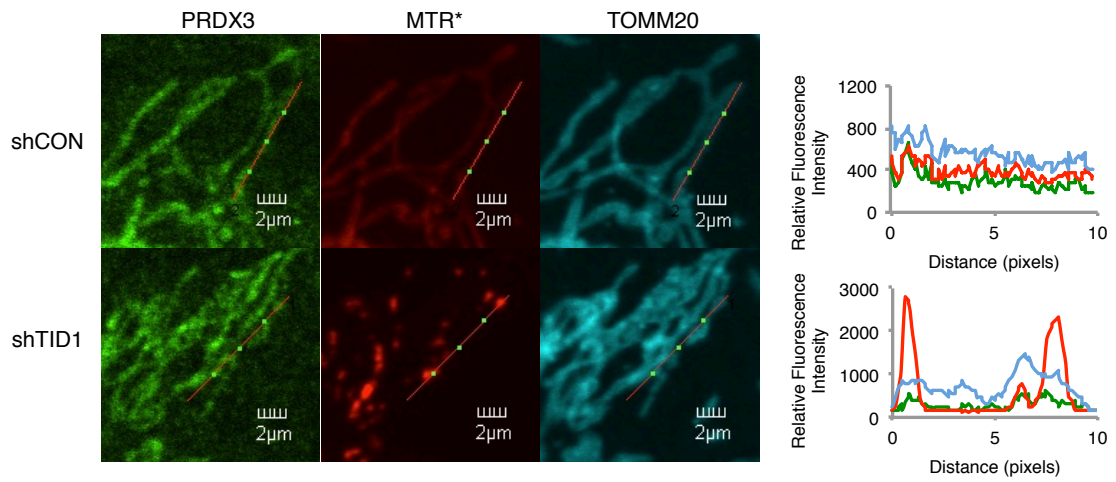


Fig S3.8: The mitochondrial matrix protein, PRDX3, does not colocalize with hyperpolarized regions. (A) Western Blot analysis using NDUFS3 antibody (top panel) and GFP antibody (bottom panel) of whole cell extract from cells treated without or with lentivirus expressing NDUFS3-eGFP. (B) Fluorescence micrographs showing siCON and siTID1-treated cells stained for PRDX3, MTR and TOMM20. Line scan analysis is shown on the right.

matrix (Fig S3.8B). Whereas NDUFS3-eGFP and MTR staining showed complete overlap with TOMM20 signal in control cells, in TID1-knockdown cells, NDUFS3-eGFP protein was concentrated at hot spots but not with TOMM20 (Fig 3.3A, line scans), suggesting that accumulation of complex I is required for hot spot formation.

We next tested whether or not respiratory activity is required for hot spot formation using 143B Rho0 cells, which lack mtDNA and thus do not express mtDNA-encoded components of the electron transport chain required for respiration. As it did in U2OS cells, TID1 silencing produced hot spots in the parental 143B cell line but did not in the 143B Rho0 cells (Fig 3.3B), despite efficient knockdown of TID1 (Fig 3.3C). We next performed CCCP washout experiments to determine if the generation of hot spots requires a functional respiratory chain. We treated control or TID1-silenced cells with CCCP to depolarize all mitochondria. Medium containing CCCP was then replaced with medium containing rotenone, 3-nitropropionic acid, or antimycin, inhibitors of complex I, II, and III, respectively. Following CCCP washout, $\Delta\psi$ was rapidly regenerated in both control and TID1 knockdown cells (Fig 3.3D). However, when electron flow was abolished during the washout phase with antimycin treatment, hot spot formation was not restored, consistent with a requirement for a functional respiratory chain. Interestingly, hot spots were observed when either rotenone or 3-nitropropionic acid, inhibitors of complex I and complex II, respectively, was included during the washout period. In the absence of the ND1 subunit containing the quinone-binding pocket, complex I is insensitive to inhibition by rotenone [38]. Given that reducing equivalents can enter the ETC at either complex I or II, these data are consistent with the notion that rotenone-insensitive complex I activity is required for hot spots in cells lacking TID1.

The spread of $\Delta\psi$ requires an uninterrupted inner membrane across a mitochondrion [39, 40]. Given that a hot spot may represent a physical obstruction to $\Delta\psi$ transmission, we tested whether mitochondria in TID1-knockdown cells are electrically coupled over a hot spot. To this end, we loaded mitochondria with TMRE, microirradiated a small region of interest (ROI) to induce focal depolarization, and assessed spreading of depolarization beyond the ROI by time-lapse microscopy [41]. In TID1-silenced cells, depolarization triggered by irradiation within the ROI spread over a hot spot as well as along the entire length of the mitochondrion (Fig 3.3E, yellow arrowhead and white arrow). Interestingly, we observed that hotspots also regained their hyperpolarity during the recovery phase (Fig 3.3E, 16''[16 s], yellow arrowhead). This suggests electrical continuity along a single mitochondrion containing a hot spot and also confirms that the machinery required to induce hyperpolarization is concentrated in these regions.

3.5.4 TID1 is recruited to oligomycin-induced hotspots and its downregulation leads to a depletion of mitochondrial DNA

Previous work has demonstrated that the mitochondrial genome in *Tid1*^{-/-} cardiomyocytes [28] is lost, which prompted us to evaluate the status of mtDNA in cells lacking TID1. Long-term (5 days) TID1 knockdown led to a reduction in mtDNA immunofluorescence signal with no effect on the intensity of TOMM20 staining (Fig 3.4A). Similarly, long-term (4 days) oligomycin treatment led to a loss of mtDNA fluorescence (Fig 3.4B). Consistent with these data, qPCR analysis revealed a 79% and 88% reduction in the mitochondrially encoded MT-RNR1 gene in cells treated with TID1 RNAi and oligomycin, respectively (Fig 3.4C). Analysis of mtDNA content and of mitochondrial function with increasing time of

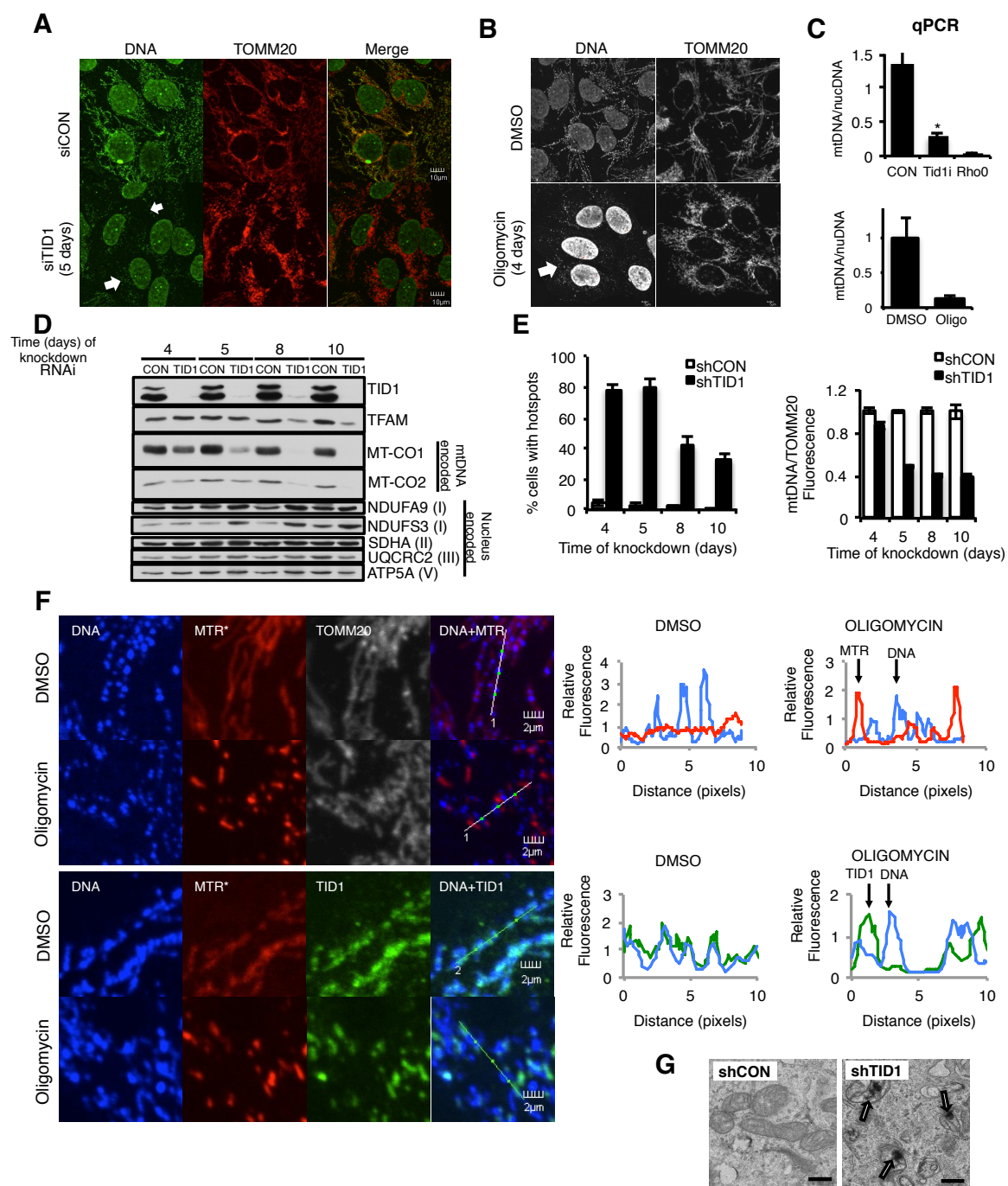


Fig 3.4: TID1 is recruited to oligomycin-induced hot spots and lead to a loss of mtDNA.

(A and B) Fluorescence micrographs showing U2OS cells treated with siRNA against CON or siRNA against TID1 (A) or oligomycin (B) and stained for DNA and TOMM20 with merged images. Arrows, mtDNA. (C) qPCR quantitation of mtDNA. Tid1i, TID1 RNAi; nuDNA and nucDNA, nuclear DNA. *, $P < 0.05$. (D) Western blot showing the time course of TID1 knockdown of ETC subunits. (E) Bar plots showing the temporal relationship between hot spot formation and mtDNA loss. (F) Fluorescence micrographs of DMSO- or oligomycin-treated U2OS cells stained for MTR (red), DNA (blue), and TOMM20 (gray, top two rows) or TID1 (green, bottom two rows). Line scans are shown on the right. MTR*, see the definition in the legend to Fig. 3. (G) Electron micrographs of cells treated with shRNA against CON or shRNA against TID1. Arrows, electron-dense bodies (for shRNA against CON, $n = 68$ mitochondria; for shRNA against TID1, $n = 100$ mitochondria). Bars = 500 nm.

knockdown revealed a mild decrease in oxygen consumption after 4 days of knockdown (Fig S3.9A), 6 days before depletion of two mitochondrion-encoded subunits, MT-CO1 and MT-CO2 (Fig 3.4D), and a loss of oxygen consumption (Fig S3.9B). In TID1-knockdown cells we observed a mild increase in the abundance of NDUFA9 and NDUFS3, two subunits of complex I, without any changes in nucleus-encoded components of complexes II, III or ATP Synthase (complex V). By day 10 of knockdown, we observed 40% reduction in cells containing hot spots, likely due to a loss of mtDNA (Fig 3.4E). After 2 days of low-dose oligomycin treatment, we observed hotspots with normal mtDNA contents; however, mtDNA content was reduced by 88% after 4 days (Fig 3.4B-C and, data not shown). Taken together, these data show that $\Delta\psi$ hot spots occur prior to mtDNA loss and bioenergetics defects.

Given the progressing phenotype in cells treated with oligomycin, we sought to monitor mtDNA presence and localization with respect to hot spot formation. In control cells, mtDNA is localized to punctate structures along the mitochondrial network (Fig 3.4F top two rows, and line scans). However following 2 days of oligomycin treatment, hot spots were devoid of mtDNA (Fig 3.4F, bottom two rows, and line scans). Whereas TID1 and mtDNA were colocalized in control cells, TID1 protein moved to hot spots and no longer colocalized with mtDNA after oligomycin treatment (Fig 3.4F, bottom two rows, Fig S3.10). These data suggest that oligomycin treatment induces a loss of association between mtDNA and TID1 at an early time point (day 2), leading to the eventual loss of mitochondrial DNA.

We next performed electron microscopic analyses to look for ultrastructural changes induced by TID1 silencing. Cells treated with control shRNA show normal mitochondrial profiles

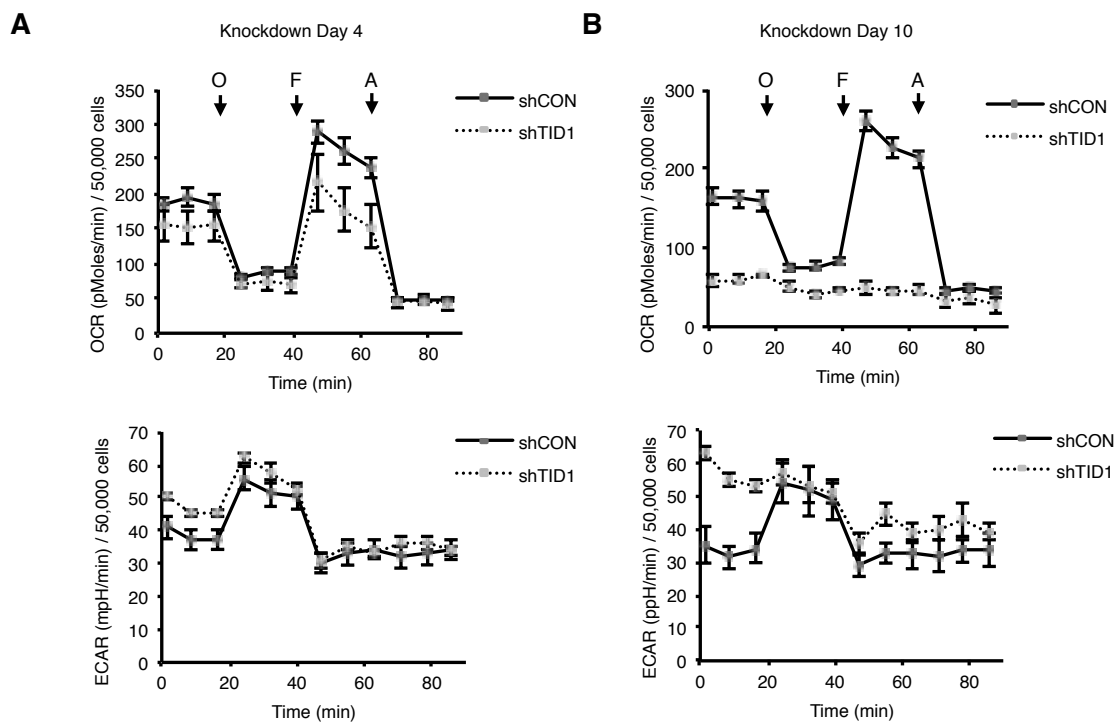
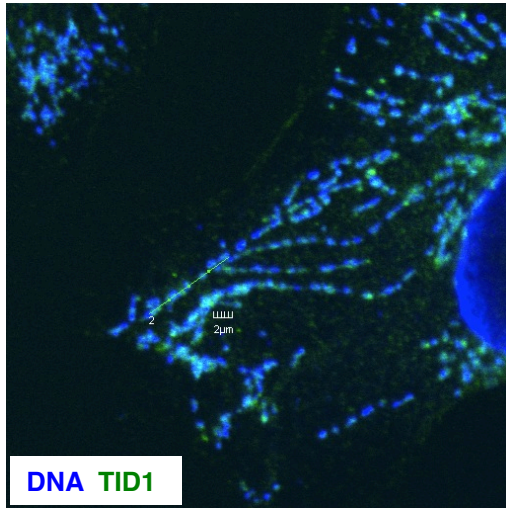


Fig S3.9: TID1-silencing leads to progressive loss of mitochondrial respiration. TID1 was silenced for 4 days (A) or 10 days (B) before measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were taken. O: oligomycin, F: FCCP, A: Antimycin A

DMSO



Oligomycin

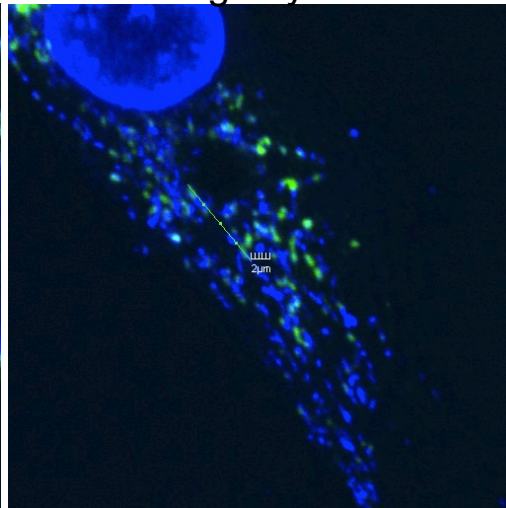


Fig S3.10: Oligomycin induces the dissociation of TID1 from mtDNA. Uncropped images that are shown in Figure 3.4F are shown here. Blue = DNA, Green = TID1.

(Fig 3.4G). In contrast, in cells depleted of TID1 protein, we observed a decrease in crista density and an accumulation of electron-dense material in ~25% of mitochondria (Fig 3.4G, arrows), consistent with the loss of respiratory capacity (Fig S3.9).

3.5.5 ATP synthase inhibition leads to a transition of mtDNA-associated soluble TID1 to insoluble TID1

Given that NDUFS3, a subunit of the respiratory complex I, is redistributed to discrete foci within the mitochondrial network, we tested whether abundance of respiratory complexes is altered in TID1-silenced cells. Blue native PAGE analysis of respiratory complexes isolated from whole cells using digitonin revealed no significant differences in the amounts of monomeric forms of complex II, III, IV, and V (Fig S3.11). In contrast, bands corresponding to supercomplexes were decreased (Fig S3.11). We also noticed that the extraction efficiencies of the complex I subunits NDUFA9 and NDUFS3 in TID1-knockdown cells were reduced (see the SDS-PAGE blot in Fig S3.11; Fig 3.5A). While these two subunits resided in the pelleted fraction in digitonin-treated TID1-silenced cells, all subunits of complexes II, III, IV and V remained soluble (Fig 3.5A). These results suggest that multiple subunits of complex I accumulate in detergent-insoluble membrane domains in TID1-silenced cells.

Consistent with the functional similarity between oligomycin treatment and TID1 silencing, oligomycin treatment also elicited the selective insolubility of complex I subunits NDUFA9 and NDUFS3 without affecting the solubility of respiratory complexes II, III, and V (Fig 3.5B). Interestingly, oligomycin treatment resulted in an increase in TID1 protein (Fig 3.5B, Fig S3.12); this additional TID1 was also recruited to a digitonin-resistant fraction. Similar

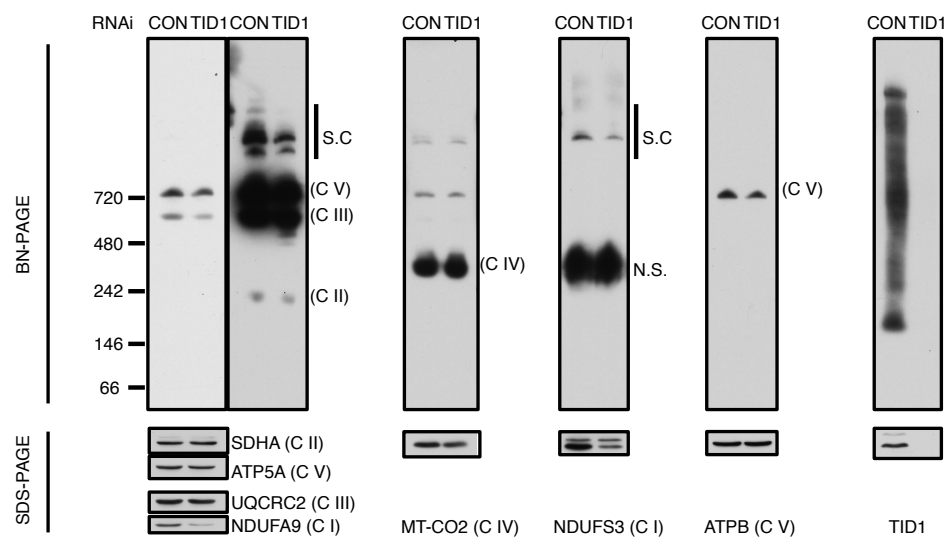


Fig S3.11: Blue Native PAGE (BN-PAGE) of mitochondrial ETC. Western blots of CON and TID1 RNAi-treated cells were extracted with 1% digitonin and separated on BN-PAGE and probed with various antibodies. From left to right: anti OXPHOS (low and high exposure), anti MTCO2, anti-NDUFS3, anti ATPB, anti TID1. SDS-PAGE blots are used as BN-PAGE input. S.C. = supercomplex (I_xIIIYIVZ), N.S. = non specific cross-reacting species.

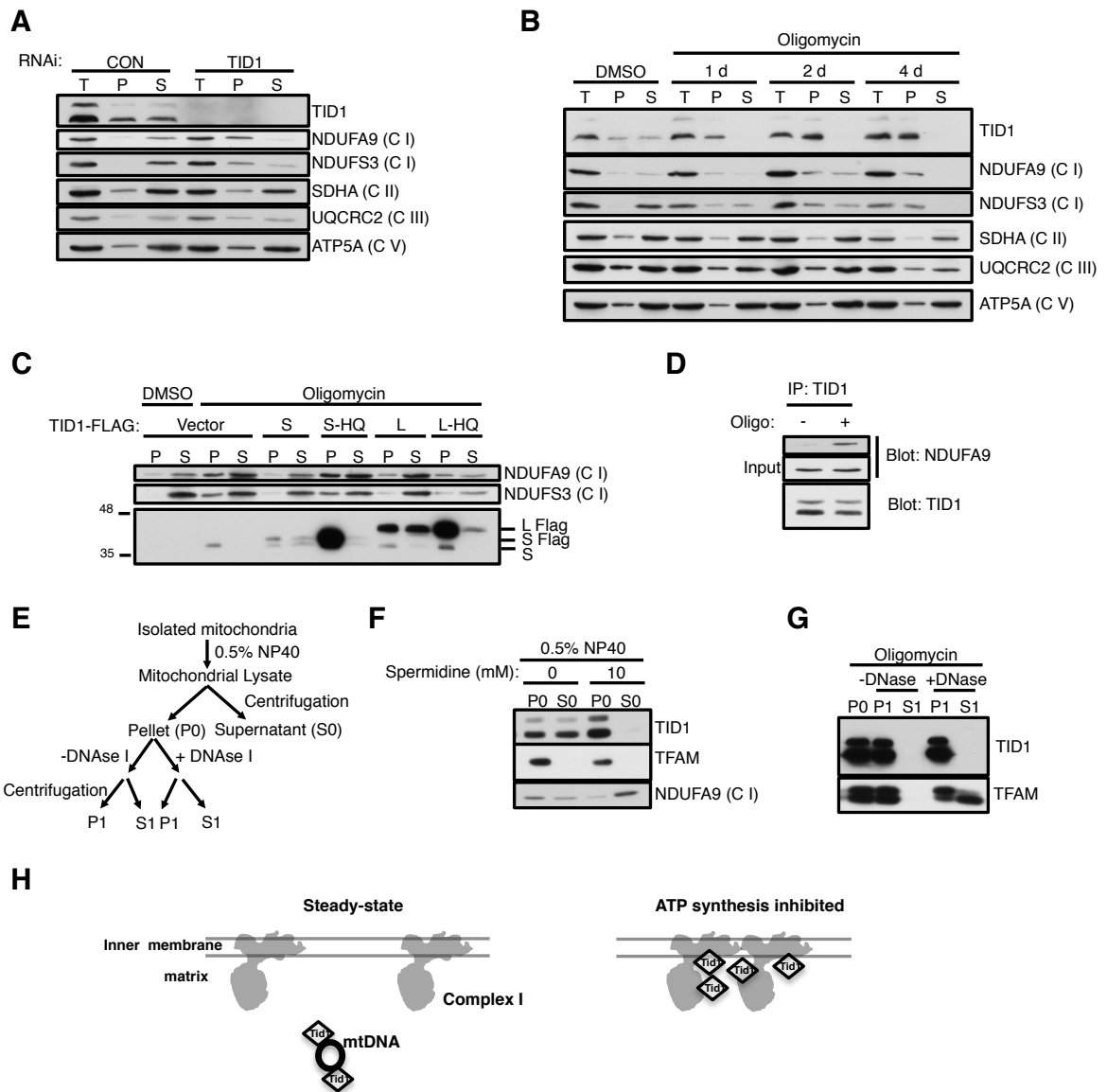


Fig 3.5: ATP synthase inhibition leads to a transition of mtDNA-associated soluble TID1 to insoluble TID1. (A) Western blot for ETC components in cells treated with shRNA against CON or TID1 and extracted with digitonin; the extracts were then fractionated into soluble (lanes S) and pellet (lanes P) fractions. The total extract (lanes T) is also included. C, complex. (B) As for panel A, but with cells treated with DMSO or oligomycin for the indicated times (d, day). (C) Western blot of cells expressing the indicated TID1 constructs and treated with oligomycin for 2 days. The results for the pellet and soluble fractions are shown. (D) Western blots showing coimmunoprecipitation of TID1 with NDUFA9. IP, immunoprecipitation. (E) Experimental schematic of the results in panels F and G. (F) Western blot of mitochondrial extracts in NP-40 separated into P0 and S0 fractions. (G) Western blot for TID1 and TFAM from the DNase I release assay. (H) Proposed model of the TID1-mediated stress response when ATP synthesis is inhibited with oligomycin. Under steady-state conditions, TID1 can be found to be associated with mtDNA. When ATP synthesis is inhibited, TID1 is recruited to a detergent-resistant fraction together with aggregates of complex I, and mtDNA is lost.

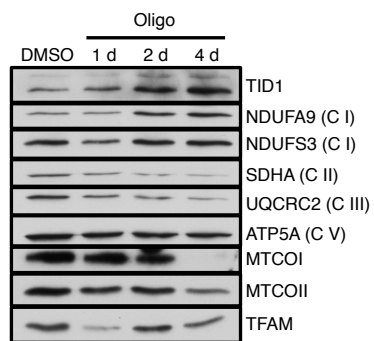


Fig S3.12: Time course analysis of TID1 and respiratory complex subunits in oligomycin-treated cells. Western blot analysis of cells treated for the indicated time with DMSO or Oligomycin. Blots were probed with the indicated antibodies.

results were obtained when cells were lysed with NP-40 or when isolated mitochondria were used as starting material (data not shown). Taken together, these data suggest that the accumulation of insoluble TID1, which is normally present in limiting amounts, is part of a stress response to inhibition of ATP synthase. Overexpression of Flag-tagged TID1 wild type but not the H121Q mutant resolubilized complex I aggregates induced by oligomycin, indicating that TID1 was indeed a limiting factor in oligomycin-treated cells (Fig 3.5C). Finally, NDUFA9 and endogenous TID1 form a precipitable complex in cells that accumulated following oligomycin treatment (Fig 3.5D).

Given that TID1 is required to maintain mtDNA fidelity, we next sought to determine if TID1 is associated with mtDNA (Fig 3.5E). Mitochondrial extracts solubilized with NP-40 were treated without or with spermidine, a polyamine used to precipitate mtDNA [42], prior to centrifugation (Fig 3.5F). The resulting fractions P0 and S0 were solubilized in SDS lysis buffer and subjected to SDS-PAGE/Western blotting. Spermidine treatment resulted in precipitation of TID1, suggesting that the TID1 protein in the soluble (S0) fraction is associated with mtDNA. We next treated the P0 fraction with DNase I to degrade mtDNA in order to determine if insoluble TID1 is associated with DNA. Following DNase I treatment, mitochondrial transcription factor A (TFAM), a well-characterized protein component of mitochondrial nucleoids, was released into the supernatant. In contrast, TID1 remained in the detergent-insoluble fraction, suggesting insoluble TID1 is not associated with DNA (Fig 3.5G).

3.6 Discussion

In this report, we show that TID1 plays a critical role in maintaining a homogeneous distribution of mitochondrial membrane potential and the integrity of mtDNA. It has been proposed that TID1 and mortalin represent an Hsp40-Hsp70 chaperone system in mitochondria based on the following experimental observations: 1. Mortalin is the only known mitochondrial Hsp70 in mammalian cells. 2. TID1 and mortalin can be found in their respective immunoprecipitates [22]. 3. TID1 can increase the ability of mortalin to disaggregate a substrate *in vitro* [29]. Here we demonstrate that maintenance of normal distribution of $\Delta\Psi$ requires the functional J-domain of TID1, indicating that TID1-mortalin regulates $\Delta\Psi$. Indeed, mortalin, like TID1, is also recruited to hyperpolarized regions following oligomycin treatment, and this recruitment is abolished when TID1 protein is depleted (data not shown). However, as silencing of TID1 or mortalin gave distinct phenotypes (hot spots compared to more global mitochondrial dysfunction), we propose that these two proteins have overlapping but distinct biological functions.

A number of studies have shown that TID1-S and TID1-L isoforms may have unique biological roles. Specifically, overexpression of TID1-S and -L elicits opposing effects on stimulus-triggered apoptosis [22] and only TID1-S clusters acetylcholine receptors, leading to efficient synaptic transmission in neuromuscular junctions [26]. Our data provide evidence that in mitochondria, TID1-S and TID1-L isoforms are also functionally distinct. Reconstitution of TID1-S but not TID1-L in TID1-silenced cells restored the uniform distribution of $\Delta\Psi$. TID1-L contains a 33-amino-acid region in its C terminus not found in TID1-S, which may compromise its capacity to regulate $\Delta\Psi$. At higher levels of expression, TID1-S and TID1-L proteins display subtle differences in their mitochondrial localization, which may help to explain this functional difference (Fig S3.13). Interestingly, a previous

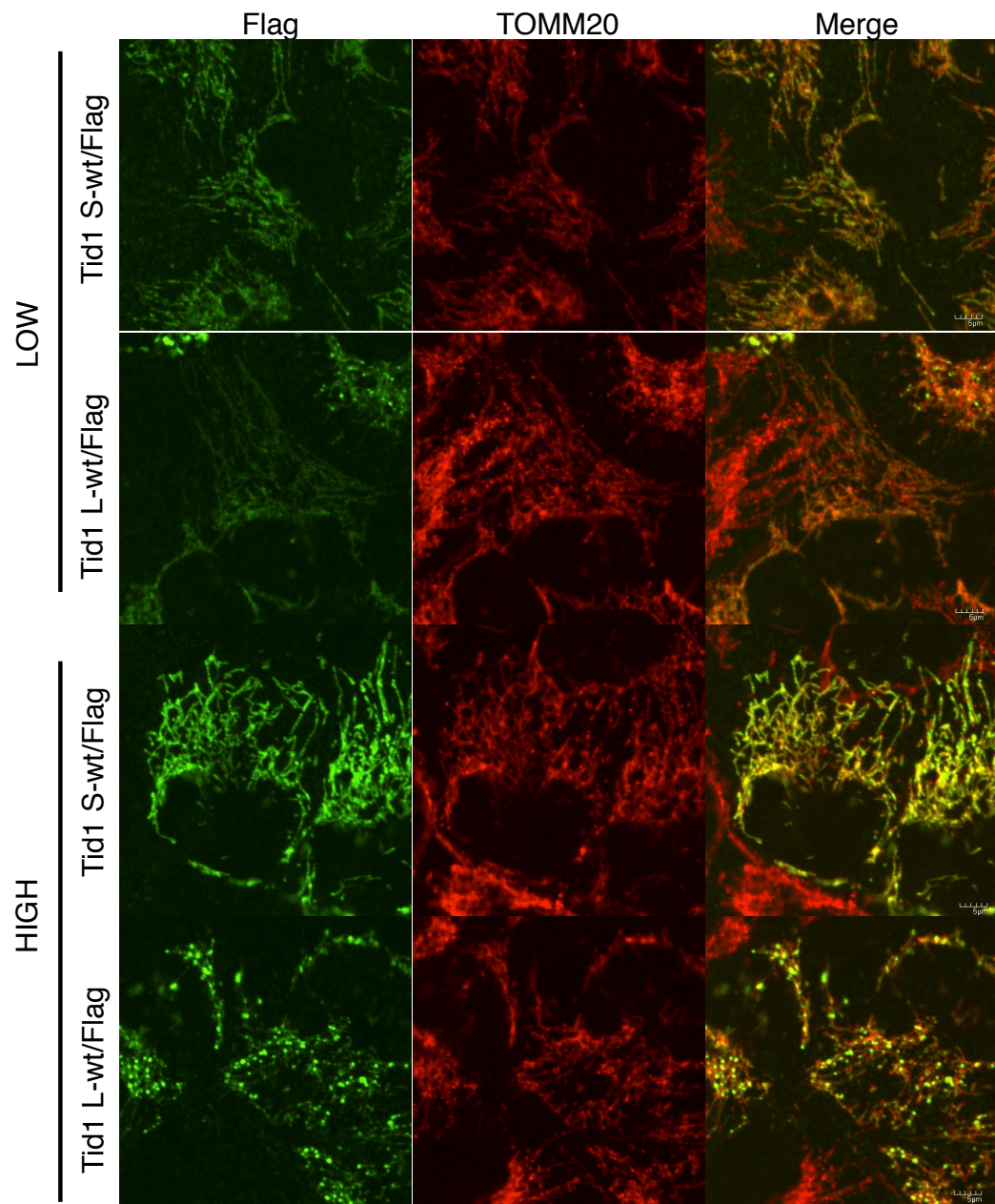


Fig S3.13: Mitochondrial localization of overexpressed TID1 S and TID1 L.

Fluorescence micrographs showing U2OS cells treated with low (top two panels) or high (bottom two panels) amounts lentivirus expressing C terminal Flag-tagged versions of TID1 S and TID1 L. Cells were stained with antibodies against Flag (green) and TOMM20 (red) antibodies.

report indicated that the N-terminal half (amino acids 1 to 222) of the TID1 protein is sufficient to induce neuronal acetylcholine receptor cluster clustering, suggesting that the C terminus is dispensable for its activity [26].

How does TID1 influence the distribution of $\Delta\psi$? We show that upon TID1 silencing, NDUFS3 adopts a punctate submitochondrial distribution, selectively colocalizing with hyperpolarized regions of the network. Both NDUFS3 and NDUFA9, two subunits of complex I, are found in a detergent-insoluble fraction, raising the possibility that aggregated complex I underlies hyperpolarization. Mitochondrial membrane potential is formed due to electron transport-coupled proton extrusion into the intermembrane space by complexes I, III, and IV, and is depleted by the activities of ATP synthase (complex V) and uncoupling proteins (UCPs). Thus, we propose that TID1 silencing leads to aggregation of complex I in submitochondrial foci resulting in hotspots in these regions, but does not affect ATP synthase or leak proteins localization.

The role of TID1 in mitochondrial DNA maintenance remains to be clarified. Disruption of *Mdj1p*, the yeast orthologue of mammalian TID1, results in a rho0-like state characterized by a lack of mtDNA [43]. In mammalian cells, TID1 copurifies in a sucrose gradient with mtDNA, suggesting that TID1 may be a component of nucleoids [44]. Our data showing that acute depletion of TID1 using RNAi does indeed lead to loss of mtDNA are consistent with the mitochondrial dysfunction, mtDNA depletion, and increased apoptosis observed in the cardiomyocyte-specific *Tid1*-knockout model [28]. When mitochondria are solubilized using nonionic detergent, the majority of mtDNA associates with the insoluble fraction (P0), with the remaining mtDNA partitioning into the (S0) fraction [45-47]. We find that TID1 protein

is similarly found in both S0 and P0 fractions and that soluble TID1 is associated with mtDNA. Given that TID1 is required for mtDNA maintenance, we suggest that TID1 in the S0 fraction contributes to overall mtDNA maintenance. The appearance of the electron-dense material bears a striking resemblance to that present in cells treated with ethidium bromide [48]. As ethidium bromide treatment selectively inhibits mitochondrial DNA polymerase [49], this observation is consistent with the idea that TID1 is required for mitochondrial DNA fidelity.

Hsp40-Hsp70 family proteins require ATP to dissociate and unfold bound substrates [50-52]. As oligomycin treatment depletes ATP in the mitochondrial compartment, we propose that TID1 becomes increasingly bound to detergent-resistant, unfolded complex I substrates (Fig 3.5G). Immunofluorescence (Fig 3.4F) and biochemical (Fig 3.5B) data support the idea that in oligomycin-treated cells, soluble TID1 is recruited to the insoluble fraction to promote the disaggregation of complex I. That the ectopic introduction of TID1 wild type but not the inactive mutant promoted disaggregation of complex I shows that endogenous TID1 is saturated with unfolded substrates when ATP levels are low (Fig S3.14) following inhibition of ATP synthase.

Interestingly, in control cells, we have noticed a rare population (<1%) of U2OS cells that display potential hot spots akin to TID1 knockdown or oligomycin-treated cells, evidence that heterogenous hyperpolarity and complex I aggregation are physiological events (Fig S3.15). Interestingly, mtDNA is also markedly decreased in these cells compared to those with a normal distribution of membrane potential. As a loss of mtDNA eventually leads to a loss of cellular proliferation and downstream cell cycle arrest [53], we speculate that mtDNA

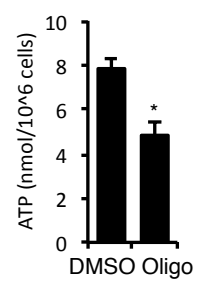


Fig S3.14. Oligomycin reduces cellular ATP levels. Bar plot showing ATP levels in DMSO or Oligomycin (2 d) treated cells * $p < 0.05$

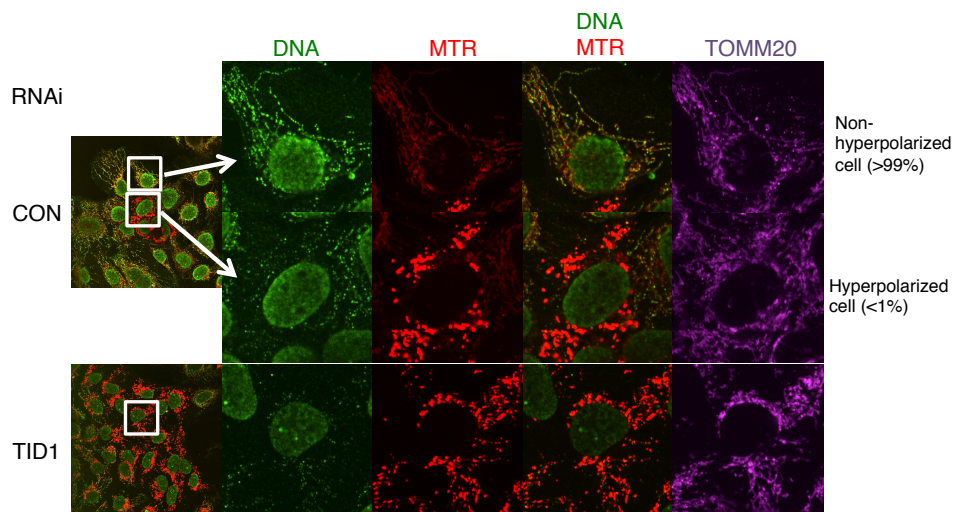


Fig S3.15. A small percentage of untreated cells display hotspots and loss of mtDNA.

(A) Fluorescence micrographs of siCON and siTID1 cells. In the siCON field, two white boxes were drawn around two cells displaying normal or heterogeneous hyperpolarized mitochondrial (hot spot) phenotype. DNA (green), MTR (red), and TOMM20 (purple) signal are shown in those cells.

depletion and loss of viability are consequences of prolonged accumulation of aggregated complex I through differential interactions with TID1 protein. Given that the accumulation of unfolded proteins and altered complex I activity are implicated in several neurodegenerative diseases [14, 20, 21], we propose that promoting TID1 activity in these contexts may be of therapeutic benefit.

3.7 Acknowledgements

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We declare that we have no conflict of interest.

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Chapter 4

ROMO1 is required for Spare Respiratory Capacity

4.1 Preface

This short chapter contains unpublished data that explores a novel role of *ROMO1* in metabolism. In our Science Signaling paper published in 2014, we showed that *ROMO1*-silencing lacked maximal respiration and spare respiratory capacity (SRC). Our lead hypothesis was that substrate supply was limited in cells lacking *ROMO1*. In this short chapter, I provide further evidence that supports a lack of substrate supply is limiting SRC. I further showed that the metabolic disturbances in *ROMO1*-silenced cells do not depend on mitochondrial fragmentation. Given the importance of (SRC) in normal physiology and disease conditions, we propose that elucidating the exact mechanism on how *ROMO1* regulates SRC will be of great therapeutic value.

Author list

Andy Cheuk-Him Ng, Matthew Norton, Robert A. Screaton

Author contribution

AC-HN designed and performed all the experiments listed in this chapter. MN discovered the SRC phenotype in *ROMO1*-silenced cells. RAS provided invaluable ideas and supervised the study.

4.2 Abstract

Reactive Oxygen Species Modulator 1 (*ROMO1*) was recently shown to mediate mitochondrial fusion by maintaining OPA1 function. Herein, we showed that *ROMO1* plays a crucial role in maintaining spare respiratory capacity (SRC), a parameter that reflects the ability of cells to increase energy output in response to stress. SRC was restored in *ROMO1*-silenced cells when excess substrates were provided. Mitochondrial pyruvate carrier inhibition selectively depleted basal respiration in *ROMO1*-silenced cells but had no effect on control cells, suggesting metabolic inflexibility in cells lacking *ROMO1*. SRC loss was not due to mitochondrial fragmentation or electron transport chain dysfunction in cells lacking *ROMO1*. In future work, we will take a metabolomics approach and utilize isotopomer labeling-LC/MS to determine the precise metabolic flux disturbances in cells lacking *ROMO1*.

4.3 Introduction

The capacity of mitochondria to increase energy output when confronted with various stressors is of fundamental importance in cell biology and physiology. The recently coined term “spare respiratory capacity” (SRC) reflects the ability of mitochondria to increase energy output due to stress and is defined as the difference between maximal respiration and basal respiration [1-3]. In pancreatic beta cells, glucose stimulates mitochondrial ATP production, leading to insulin release [4]. In heart, catecholamines stimulate ATP production required for heart contraction [5]. In neurons, the maintenance of plasma membrane potential and release of neurotransmitters increase the activity of various ATPases and therefore increasing energy demand [6, 7]. The inability of mitochondria to meet these metabolic demands can lead to energy crisis and cell death [3, 8].

SRC also regulates various aspects of pathological conditions. The ability of tumor cells to increase their biomass is associated with various ATP-dependent biosynthetic reactions [9]. Cytokine-mediated maintenance and establishment of CD8⁺ memory T cells and neuronal survival after ischemic stroke-induced glutamate excitotoxicity critically depend on this SRC [10, 11].

Mitochondria exist in cells as an interconnected reticulum sculpted by fission and fusion reactions, collectively known as mitochondrial dynamics. Reactive Oxygen Species Modulator 1 (ROMO1) is a novel inner mitochondrial membrane protein that couples oxidative stress to mitochondrial fusion machinery [12]. ROMO1 is required for the stability of long isoforms of OPA1 and oligomers, both required for mitochondrial inner membrane

fusion and maintenance of cristae folds. In addition, oxidative signals inactivate ROMO1 by stimulating its oligomerization via disulphide bridges.

Herein we showed that ROMO1 maintained SRC in cultured cells. We provided evidence that an inability to utilize an unidentified substrate underlies the SRC defect in *ROMO1*-silenced cells.

4.4 Methods and Materials

4.4.1 Cell Culture and Reagents

U2OS cells were obtained from ATCC and cultured in DMEM + 10% FCS + antibiotics in a humidified atmosphere with 5% CO₂. Mitochondrial pyruvate carrier (MPC) inhibitor UK5099 was from Sigma (PZ0160). Antibodies: Anti-ROMO1 (2C12, 1:2000) was from Origene. Anti-OPA1 (1:1000) was from BD Transduction Laboratories.

4.4.2 RNA interference and cDNA plasmids

SiRNA targeting human *ROMO1* were as follows: CUGCUUCGACCGUGUCAAA. Negative Control siRNA was from Qiagen. Cells were transfected with siRNA for 72 h.

4.4.3 SDS-PAGE and Western Blotting

SDS-PAGE and immunoblot analysis were performed as previously described [13].

4.4.4 Extracellular Flux Assay

Measurements of mitochondrial oxygen consumption (OCR) were performed as described [12]. Briefly, 6500 U2OS cells were transfected with siRNA targeting *ROMO1* or non-silencing control on Seahorse XF24 cell culture microplate on day 0. On day 3, cells were rinsed and media was replaced with pre-warmed, XF base media (pH 7.2). XF base media

was not supplemented unless otherwise indicated. Cells were equilibrated at 37°C in an incubator without CO₂ for 1 h before the culture plate was placed in XF24 analyzer. Final concentrations of drugs were as follows: 0.5 µM oligomycin, 1.0 µM FCCP, 0.5 µM antimycin. Protein was quantified using BCA (Thermo Scientific) for normalization.

4.4.5 Metabolomics

1.5 X 10⁵ U2OS cells per condition were transfected with siRNA targeting ROMO1 or non-silencing control in 6-well plates. 72 h post-transfection, cells were rinsed with 2mL of G0 media (DMEM + 0 mM glucose, 3.7 g/L NaHCO₃, 10 mM HEPES, 0.2 % FFA-free BSA, and 4 mM glutamine). Cells were incubated with 1 mL of G5 (G0 + 5.5 mM glucose) for 2 h for cells to reach metabolic steady state. Then cells were rinsed with 2 mL of G0, and incubated with 1 mL of ¹³C-G5 (G0 + [U-¹³C₆] glucose for 0, 5, 15, 30, 60, and 120 min. Cells were rinsed with 2 mL of ice cold PBS and quenched with 150 µL of quenching buffer (20% MeOH, 0.1 % formic acid, 10 µM D4-taurine, 3 mM NaF, 1 mM phenylalanine, 100 µM EDTA). Cells were scraped and transferred to 96-well LC/MS/MS V-bottom polypropylene plate (BD #353263) on dry ice. Samples were stored at -80°C prior to LC/MS/MS analysis.

4.4.6 Statistical analyses

All results were obtained through a minimum of three independent experiments. Results are reported as mean ± s.e.m. Statistical significance for all data was determined using two-tailed unpaired Student's *t*-test; p-values < 0.05 were considered significant.

4.4.7 Repeatability of experiments

All experiments were conducted at least twice and consistent results were obtained.

4.5 Results

4.5.1 ROMO1 is required for spare respiratory capacity

Spare respiratory capacity can be experimentally determined by performing extracellular flux measurements and determining the oxygen consumption difference between maximal respiration and basal respiration (Fig 4.1A). Basal oxygen consumption rate (OCR) of *ROMO1*-silenced U2OS cells (siROMO1) was not significantly different compared to non-silencing control (siCON) (Fig 4.1B,E). In contrast, FCCP-stimulated maximal oxygen consumption was ablated in *ROMO1*-silenced cells. There was a slight increase in basal uncoupling as evidenced by oligomycin-stimulated OCR (Fig 4.3B). When the measurement was conducted in assay medium containing either excess pyruvate or glutamine, maximal respiration was restored (Fig 4.1C-E). This suggests that in *ROMO1*-silenced cells, the utilization of metabolic substrate is impaired. FCCP and oligomycin treatment in *ROMO1*-silenced cells decreased and increased membrane potential ($\Delta\psi_m$), respectively and in a dose-dependent manner, suggesting the directionality of electron flow and uncoupler sensitivity were not different compared to control cells (Fig S4.1). Cellular ATP was not different between control and *ROMO1*-silenced cells (Fig S4.2).

4.5.2 *ROMO1*-silenced cells are sensitive to inhibition of pyruvate metabolism

Mitochondria utilize various extracellular and intracellular substrates depending on its intra- and extracellular availability. Glucose-derived pyruvate, fatty acids, and amino acids (glutamine/glutamate) are amongst the most common substrates used for mitochondrial respiration [14, 15]. In control cells, treatment with mitochondrial pyruvate carrier (MPC) inhibitor, UK5099, had no effect on mitochondrial respiration (Fig 4.2A-B). Whereas,

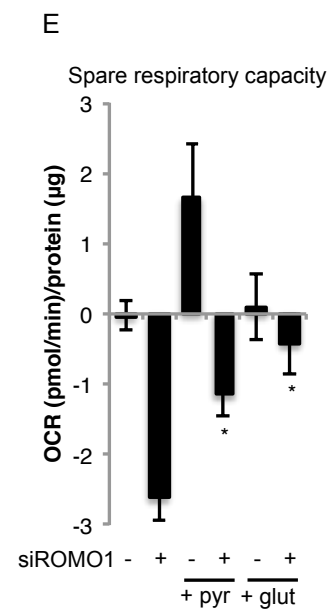
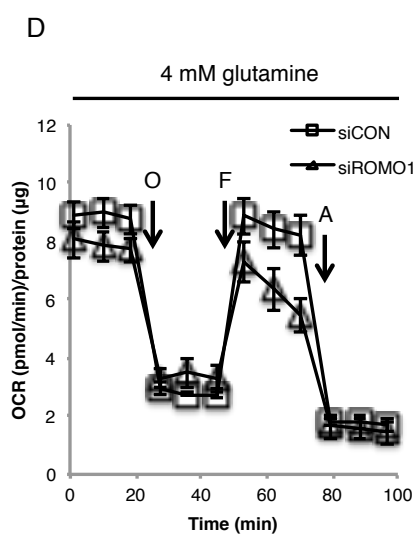
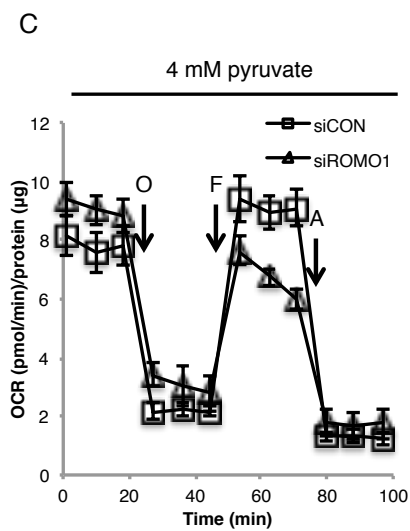
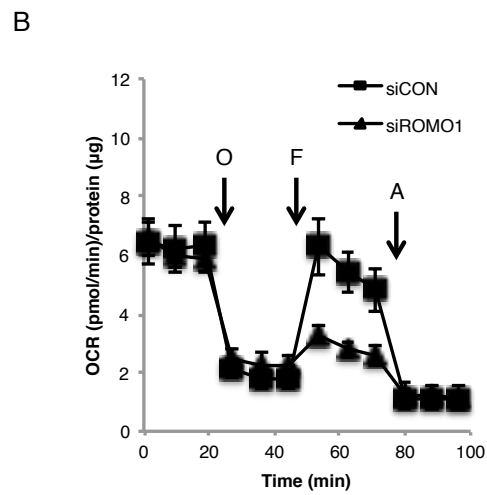
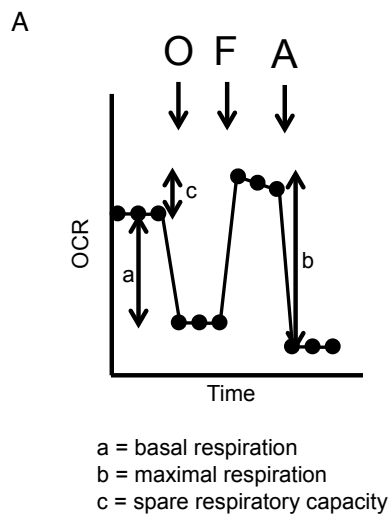
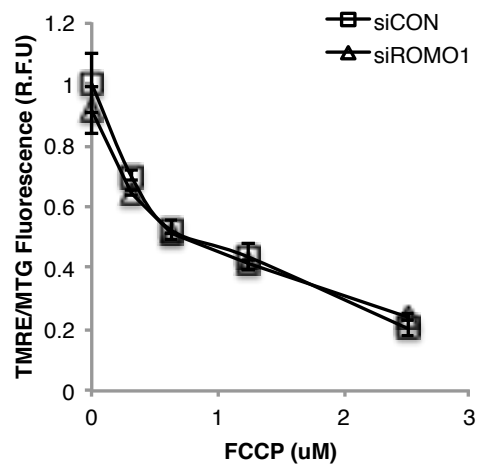


Fig 4.1: ROMO1 is required for spare respiratory capacity. (A) Schematic of extracellular flux assay to measure oxygen consumption rate (OCR). Oxygen consumption rate (OCR) was plotted against time. Oligomycin (O), FCCP (F), and antimycin (A) were injected sequentially. a = ATP turnover, b = maximal respiratory rate, c = spare respiratory capacity. (B) OCR measurement in siCON and siROMO1 cells in XF basal medium with no additional substrates. (C and D) same as (B) but assay medium containing 4 mM pyruvate or 4 mM glutamine, respectively. (E) Quantification of spare respiratory capacity from (B-D). * $p < 0.05$ relative to siROMO1 + no substrate.

A



B

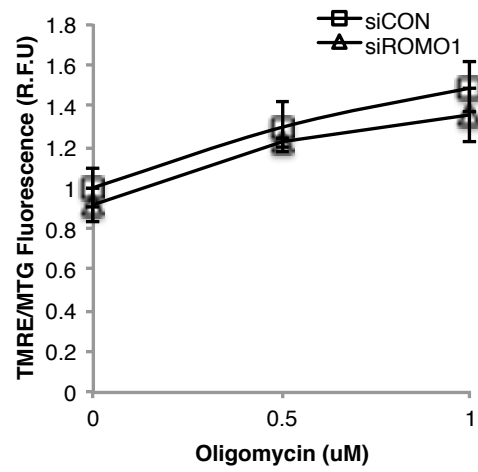


Fig S4.1: *ROMO1*-silencing does not affect the directionality of electron flow.

Fluorescence measurements of images taken with the Opera confocal microscope and quantified with Columbus software package. siCON and siROMO1 cells were loaded with 10 nM TMRE ($\Delta\psi_m$ -sensitive dye) and mitotracker green ($\Delta\psi_m$ -insensitive dye) for 45 min. Then cells were treated with indicated doses of FCCP (A) or oligomycin (B).

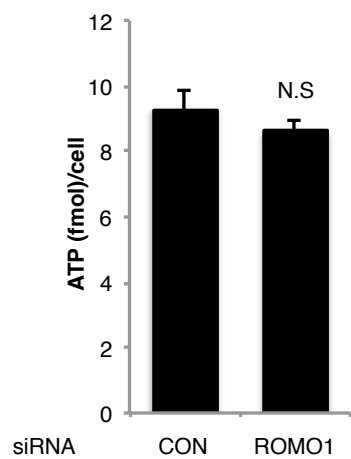


Fig S4.2: ROMO1 does not regulate basal ATP levels. ATP measurements using CellTiter-Glo Luminescent Assay. ATP standard was used to determine absolute ATP amounts. Error bars are reported as standard errors of the mean.

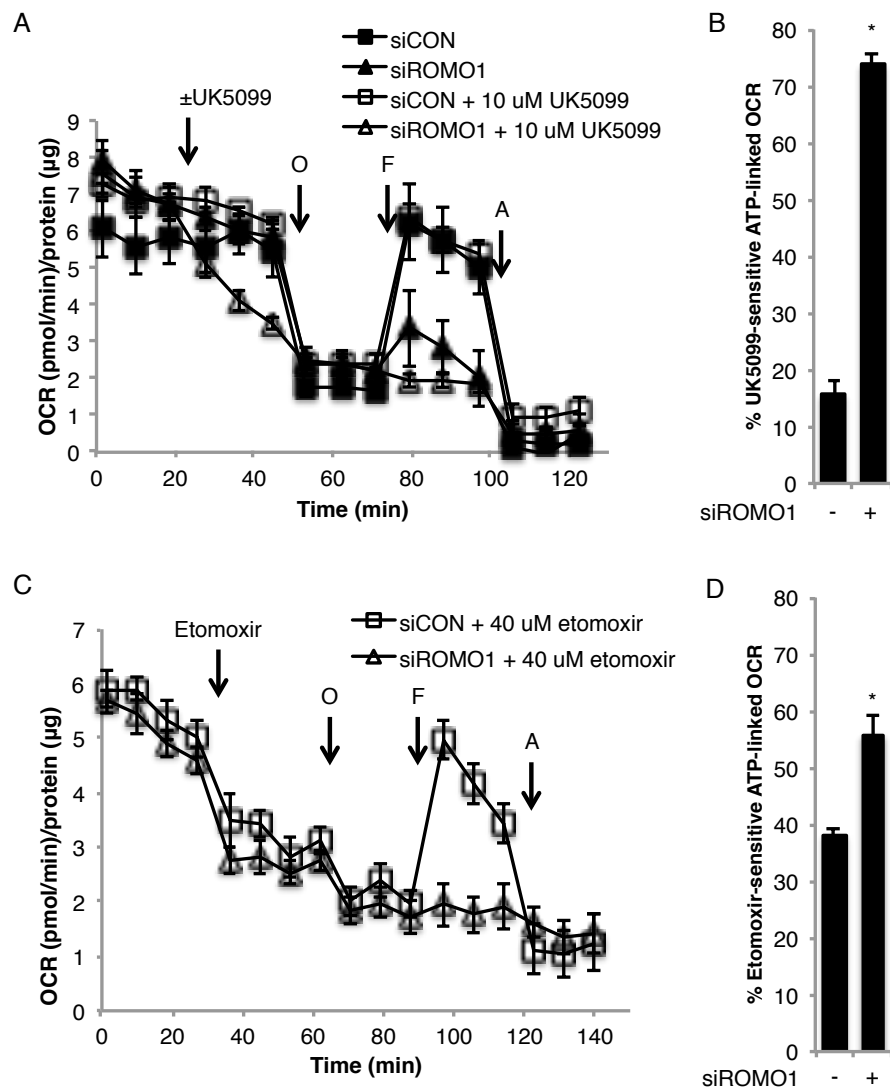


Fig 4.2: *ROMO1*-silenced cells are sensitive to inhibition of pyruvate metabolism. (A) OCR measurement of siCON or siROMO1 cells. Cells were injected without or with UK5099 after rate 3. Note that after UK5099 treatment, siROMO1 loses OCR, whereas OCR in siCON cells was maintained. **(B)** Quantification of data from (A). **(C)** same as (A), but etomoxir was used. **(D)** Quantification of data from (C). UK5099 sensitive ATP-linked OCR was calculated by the following formula: $(100\% - 100\% \times [\text{rate 6} - \text{rate 7}] / [\text{rate 3} - \text{rate 7}])$. Etomoxir-sensitive ATP-linked OCR was calculated by the following formula: $(100\% - 100\% \times [\text{rate 8} - \text{rate 9}] / [\text{rate 4} - \text{rate 9}])$. * $p < 0.05$ relative to siCON + inhibitor.

UK5099 treatment significantly depleted OCR in *ROMO1*-silenced cells, suggesting a high dependence on pyruvate oxidation in maintaining basal metabolism. Treatment with the fatty acid oxidation inhibitor, etomoxir, only slightly enhanced inhibition of basal respiration in *ROMO1*-silenced cells (Fig 4.2C-D). Therefore, control and *ROMO1*-silenced cells depended on fatty acid oxidation for basal respiration to a similar degree. Together these results showed that unlike control cells, *ROMO1*-silenced cells were unable to meet their energetic demands by switching utilization to fatty acids and glutamine after Mpc inhibition.

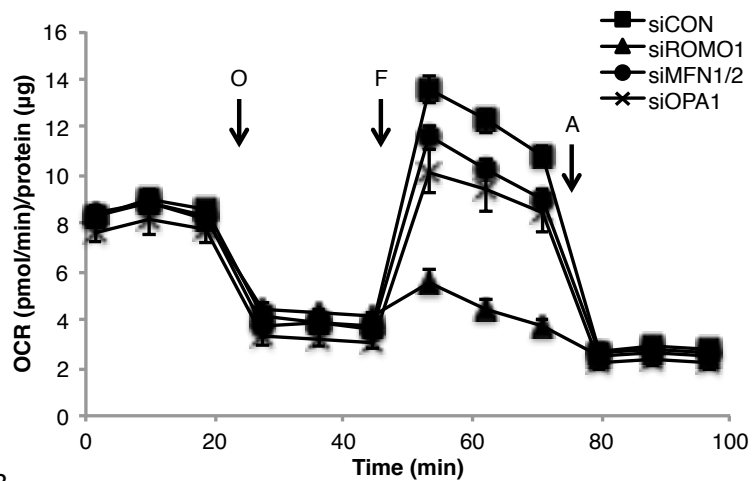
4.5.3 Spare Respiratory Capacity does not depend on mitochondrial fusion

ROMO1-silencing resulted in mitochondrial fragmentation at least in part due to loss of OPA1 oligomers and increased processing from OPA1-L to OPA1-S isoforms [12]. We tested whether mitochondrial fragmentation underlie the loss of SRC in *ROMO1*-silenced cells. Knockdown of mitofusins (*MFN1/2*) or *OPA1* results in extensive mitochondrial fragmentation [16, 17]. However, *MFN1/2*- and *OPA1*-silenced cells largely maintained SRC, suggesting SRC and mitochondrial fragmentation in *ROMO1*-silenced cells were epiphenomenon (Fig 4.3).

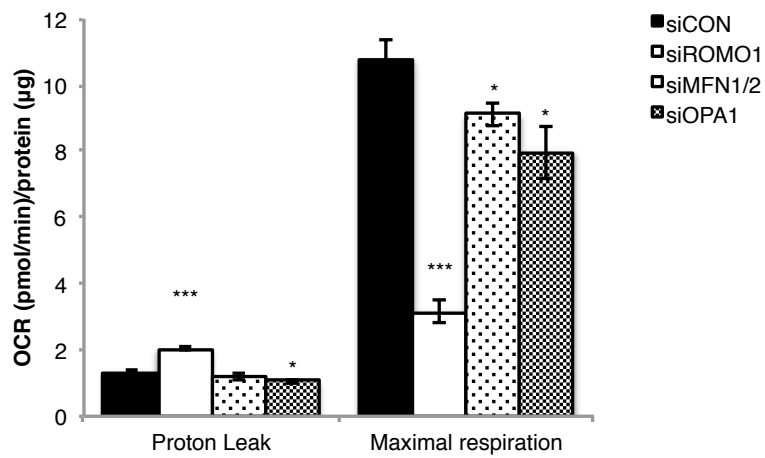
4.5.4 ROMO1 does not affect the function of electron transport chain

Electron transport chain (ETC) dysfunction can limit basal or maximal oxygen consumption rates. To test if this underlies the loss of SRC in *ROMO1*-silenced cells, we performed extracellular flux measurements on plasma membrane-permeabilized cells to enable direct mitochondrial substrate utilization. Cells were treated with recombinant perfringolysin (rPFO/XF PMP) to selectively permeabilize the plasma membrane while leaving mitochondria intact [18]. Pyruvate or glutamate, succinate, and TMPD were used drive

A



B



C

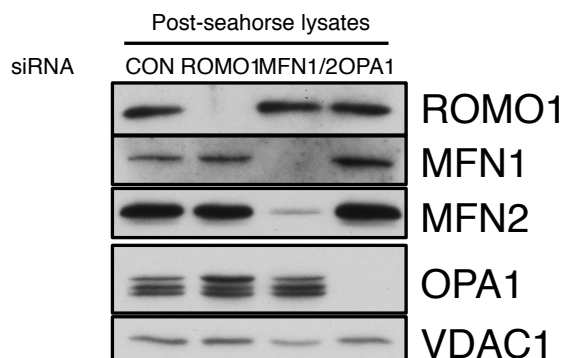


Fig 4.3: Spare respiratory capacity does not depend on mitochondrial fusion. (A) OCR measurements of siCON, siROMO1, siMFN1/2, and siOPA1. **(B)** Quantification of proton leak (rate 4 – rate 9) and maximal respiration (rate 7 – rate 9) from (A). * $p < 0.05$, *** $p < 0.0001$ relative to siCON. **(C)** Western blot of lysates from OCR experiment (A) showing knockdown. VDAC1 protein is used as the loading control.

complex I, II, and IV respiration, respectively. Unlike in intact cells, respiration in permeabilized siROMO1 cells given mitochondrial substrates showed no significant differences compared to permeabilized siCON cells (Fig S4.3). These data suggest no impairment in ETC function, but rather, a defect in endogenous substrate utilization.

4.6 Discussion

ROMO1 is indispensable for normal tubular mitochondrial network through its effect on OPA1 [12]. In this work, we performed detailed extracellular flux analysis to show that *ROMO1* has a critical role in metabolism. Specifically, SRC and maximal respiration, but not basal respiration, required the presence of *ROMO1*.

The maximal respiration rate depends on substrate uptake and oxidation rates, and electron transport chain function [8]. The addition of the uncoupler FCCP allows for the unrestricted return of protons from intermembrane space to the matrix, therefore resulting in maximum rates of electron flow from substrates to molecular oxygen. We have previously performed a titration of FCCP to ensure that the dose used results in maximal respiration but does not completely deplete $\Delta\psi_m$, which could inhibit mitochondrial substrate import, acidify cytoplasm and also result in mitochondrial Ca^{2+} release [8], all of which impact respiration. Theoretically, the true maximal rates can be measured by activating non-mitochondrial ATPases in intact cells. However, this may not always be practical and is fraught with caveats [8]. Activating cellular ATPases may be possible in neurons by activating plasma membrane Na⁺K⁺ ATPases [6, 7]. However, it must be kept in mind that the increase in ATPase may not fully activate maximal ETC activity.

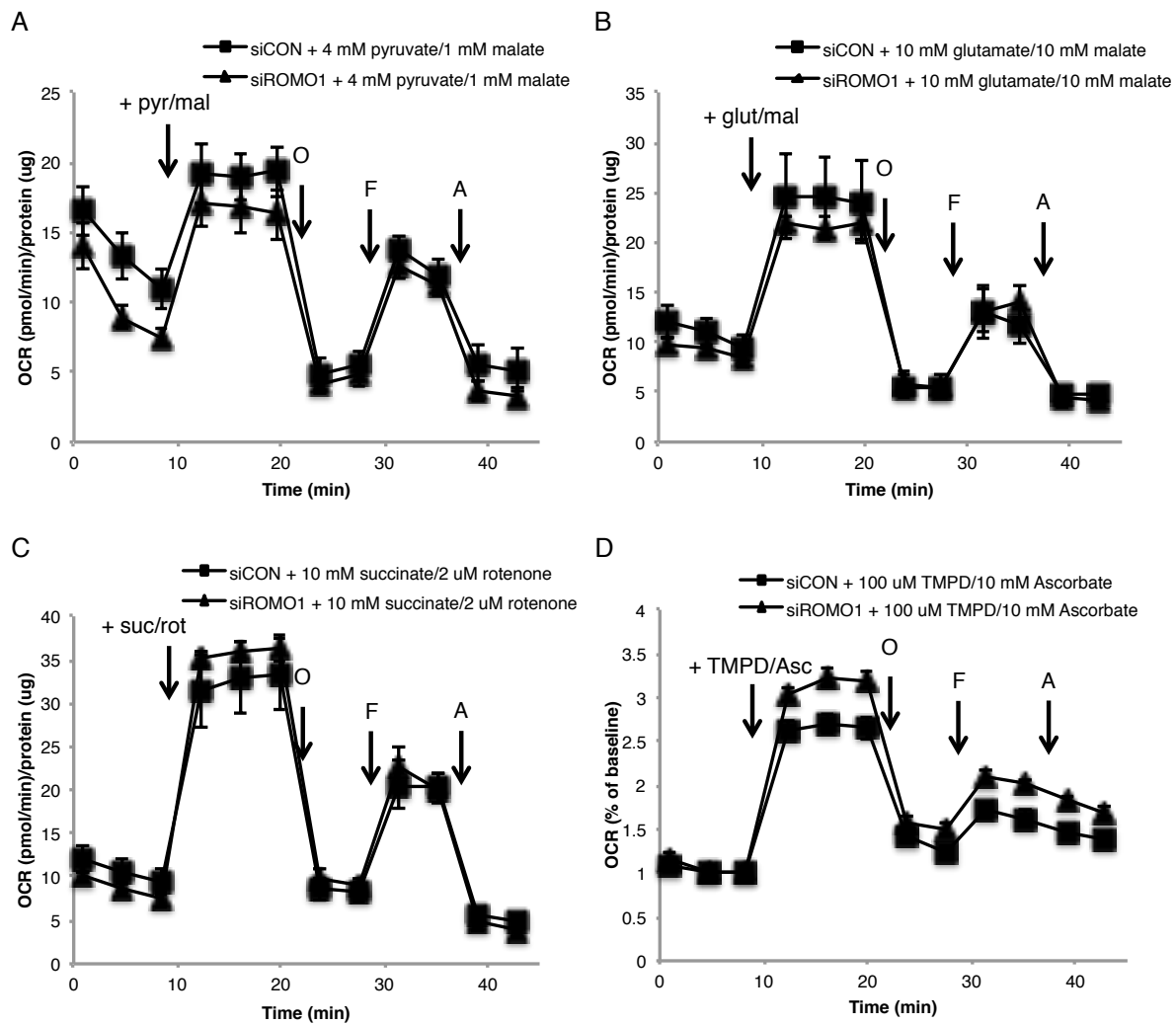


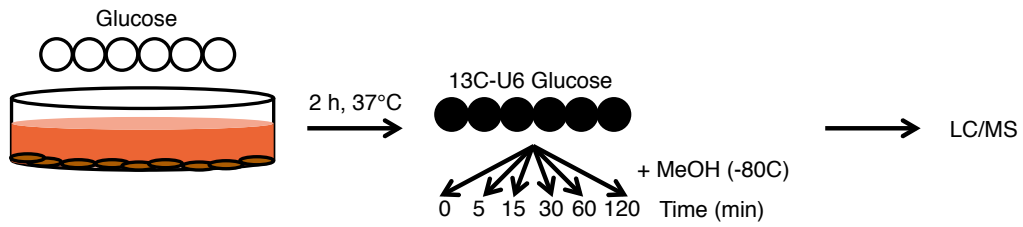
Fig S4.3: ROMO1 does not regulate ETC function. OCR measurements in siCON and siROMO1 cells treated with 1 nM recombinant perfringolysin (rPFO), purchased from seahorse biosciences. Indicated substrates were provided in the first injection. Pyr/mal = pyruvate/malate; suc/rot = succinate/rotenone; TMPD/Asc: *N,N,N',N'*-tetramethyl-*p*-phenylenediamine/ascorbate. Representative data from at least three independent experiments were shown.

The observation that excess exogenous pyruvate and glutamine restored maximal respiration rates suggests substrate supply was limiting maximal respiration rates in *ROMOI*-silenced cells (Fig 4.1). We tested the TCA and ETC function by using permeabilized cells and supplying mitochondrial fuels that feed into complex I, II, and IV. We showed no change in TCA/ETC function in this experimental paradigm (Fig S4.3).

Basal respiration, in general, is controlled mainly by cellular ATP demand. Hence, the term “ATP turnover rate” reflects the difference between basal respiration and respiration after oligomycin treatment. Substrate availability may also limit basal respiration. This is in contrast to using isolated mitochondria or permeabilized cells, where substrate and ADP are provided in excess, and therefore respiration rates are not limited by substrate supply. Etomoxir lowered basal respiration of both control and *ROMOI*-silenced cells to a similar extent, suggesting basal rates in both genetic conditions depended partially on fatty acid metabolism. In contrast, UK5099 treatment inhibited basal rates of *ROMOI*-silenced cells but not control cells, suggesting an indispensable role of pyruvate oxidation for basal respiration rates in *ROMOI*-silenced cells. Therefore the use of UK5099 unmasked a role of substrate supply in limiting basal respiration in *ROMOI*-silenced cells.

Based on the above extracellular flux results, we hypothesize that loss of *ROMOI* led to altered metabolic flux, leading to a cellular condition where substrates were limiting. Future work using isotopomer labeling and liquid chromatography/mass spectrometry will identify which metabolic pathways are altered in *ROMOI*-silenced cells (Fig S4.4A). Briefly, control or *ROMOI*-silenced cells will be cultured in medium containing 5.5 mM unlabeled glucose until metabolic steady state. Cells will then be cultured in medium containing ^{13}C -U6 glucose. Labeled glucose will enter the cell and labeled carbons

A



B

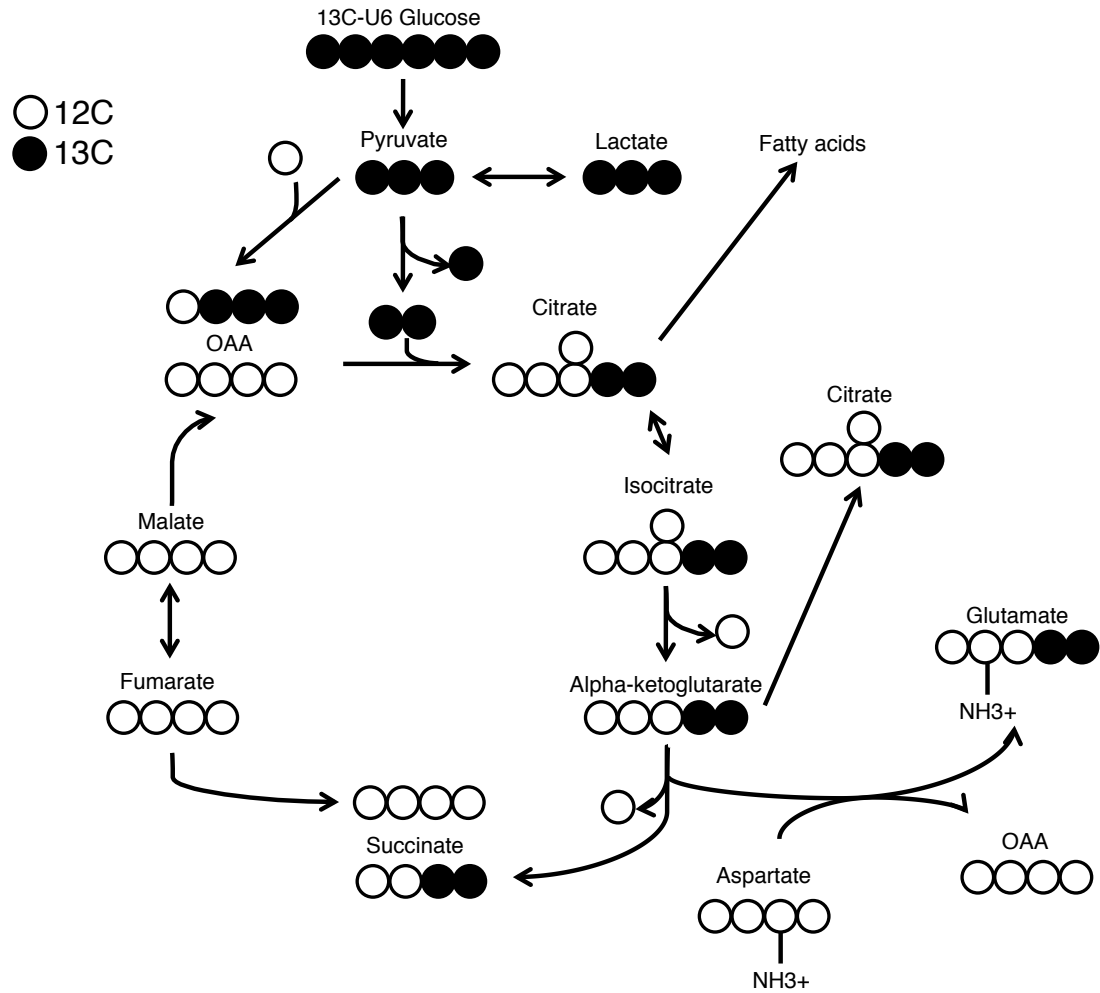


Fig S4.4: Schematic of isotopomer-labeling experiment. **(A)** Brief protocol of isotopomer-labeling experiment. Cells will be cultured in medium containing unlabeled glucose for 2 h until metabolic steady state is reached. Then cells will be provided ^{13}C -U6 glucose, which enters the cell and is metabolized via glycolysis and TCA. Cell extracts will be collected at various indicated timepoints. LC/MS will be performed in Dr. Richard Kibbey's lab (Yale University). **(B)** Schematic of glucose metabolism showing how labeled glucose carbons incorporate into various TCA and non-TCA metabolites.

will be incorporated into various metabolites. Cell extracts will be collected at various time points to allow measurements of metabolic flux until new metabolic steady state is reached. In collaboration with Dr. Richard Kibbey (Yale University), we will measure steady-state metabolites level and amount of carbon-13 incorporation in various metabolites. Depending on cell type, glucose-derived carbons may be shunted away from the canonical oxidation pathway (TCA cycle) (Fig S4.4B). For example, in response to a high energy charge, glucose may enter TCA via citrate, but exported out of mitochondria for fatty acid synthesis. Also in transformed cells, various glucose-derived TCA metabolites can be used for various biosynthetic reactions. We hypothesize that isotopomer labeling will allow us to detect any metabolic flux disturbances in cells lacking *ROMO1*.

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Chapter 5

Homozygous mutations in MFN2 cause Multiple Symmetric Lipomatosis associated with Neuropathy

5.1 Preface

This chapter contains a published paper (Sawyer et al, *Human Molecular Genetics*, 2015) showing homozygous *MFN2* mutations cause a condition called multiple symmetric lipomatosis (MSL). Using whole-exome sequencing, Dr. Kym Boycott and Dr. Sarah Sawyer discovered this mutation in two brothers with MSL. We identified a third MSL patient in Dr. Garth Nicholson's patient series with the same mutation (Sydney, Australia). My role in this project was to perform cell-based experiments to show whether this mutation confers a functional defect in the MFN2 protein. I showed that this MSL mutation affected MFN2-mediated fusion by impairing MFN2 complex formation. Permission was obtained to published patient images in this chapter.

Author list

Sarah L. Sawyer*, Andy Cheuk-Him Ng*, A. Micheil Innes, Justin D. Wagner, David A. Dyment, Martine Tetreault, Care4Rare Canada Consortium, Jacek Majewski, Kym M. Boycott, Robert A. Screaton*, Garth Nicholson*

*equal contribution

Author contribution

SLS, JDW and KMB performed whole-exome sequencing and identified the homozygous *MFN2* mutation in the two brothers with MSL. GN provided patient data for the third, unrelated patient. AC-HN designed and performed all the functional experiments (Fig 5.3) in this manuscript. AC-HN wrote the first draft of the functional data in this manuscript. RAS provided invaluable ideas and supervised AC-HN's work.

5.2 Abstract

Multiple symmetric lipomatosis (MSL) is a mitochondrial disorder with impaired brown fat metabolism that has been associated with MERFF mutations in some, but not all patients. We studied a sibling pair and an unrelated individual who presented with multiple symmetric lipomatosis (MSL) and neuropathy to determine the genetic etiology of this disorder in patients who did not carry the MSL-associated MERFF mutation. Whole exome sequencing was performed on the siblings and a rare, shared homozygous mutation in *MFN2* (c.2119C>T; p.R707W) was identified. The mutation was not present in their healthy siblings. In silico programs predict it to be pathogenic and heterozygous carriers of the *MFN2* p.R707W substitution are known to have Charcot-Marie-Tooth disease (CMT). A third, unrelated, patient with multiple symmetrical lipomatosis and neuropathy also harboured the same homozygous mutation and had been previously diagnosed with CMT. Functional studies in patient fibroblasts demonstrate that the p.R707W substitution impairs homotypic (*MFN2*-*MFN2*) protein interactions required for normal activity, and renders mitochondria prone to perinuclear aggregation. These findings show that homozygous mutations at p.R707W in *MFN2* are a novel cause of multiple symmetrical lipomatosis.

5.3 Introduction

Multiple symmetric lipomatosis (MSL, OMIM 151800) is a rare mitochondrial disorder of brown fat. Patients present in their 20-40's with cervical and thoracic lipomatosis with progressive distal weakness and neuropathy. Patients often develop diabetes later in the course of disease. A mitochondrial etiology was first suggested in 1991, when ragged red fibers (RRF) were identified on muscle biopsies of patients with MSL [1]. Since then, the m.8344A>G mutation associated with MERFF [2, 3] and mitochondrial deletions have been identified in a subset of individuals with MSL [4], and conversely multiple lipomatosis/lipomas have been described in 15-25% of patients with MERFF syndrome [5]. Familial recurrences consistent with matrilineal transmission of mitochondrial mutations have been reported. There remain, however, a large number of patients with clinically diagnosed MSL without identifiable MERFF-associated mutations suggesting that there may be other genetic etiologies of MSL. Here we report three patients with MSL, two brothers and an unrelated individual, who harbor a recurrent homozygous mutation in *MFN2*, and propose that this mutation in *MFN2* is a novel cause of MSL.

5.4 Results

5.4.1 Patient 1 and 2

Patient 1 and 2 are brothers (Fig 5.1) who developed cervical and thoracic lipomatosis in their mid 20's. Patient 1 presented with two small lipomas on the back of his neck in his 20's. Over the course of the following 25 years he developed striking unencapsulated cervical and thoracic lipomatosis and progressive tongue hypertrophy. He has undergone multiple liposuction surgeries, which were only of temporary benefit before the lesions returned. He

A



B



C



D



E



F



Fig 5.1: (A, B, C). Patient 1 at 61 years of age with cervical and thoracic lipodystrophy with decreased subcutaneous fat on arms and lower thorax down to his legs. (D) Patient 2, brother of Patient 1, shown at age 63 years; post-liposuction surgery. (E, F) Patient 3 at 60 years with cervical and thoracic lipodystrophy with decreased subcutaneous fat on legs.

has swallowing difficulties due to the bulk of the cervical lesion and tongue hypertrophy. At age 53 years, he presented with significant peripheral neuropathy, paresthesias and weakness, and required a cane to ambulate by 58 years. Nerve conduction studies and EMG demonstrated length dependent polyneuropathy with evidence of denervation/reinnervation. He is on Lyrica for neuropathic pain. He was diagnosed with diabetes mellitus (DM) in his early 50's, and is well controlled by oral hypoglycemic medication. His glucose control was improved after liposuction; fasting glucose and HbA1C were within the normal range a few weeks post-liposuction on his medications. Genetic testing for the MERFF mutation (m.8344A>G) was negative in a peripheral blood sample, as were other common mitochondrial mutations (m.3243A>G, m.3260A>G, m.3303C>T and m.8993T>G/C). Biochemical studies have repeatedly demonstrated an elevated lactate (5.02 and 4.7 mmol/L, ULN 2.2). His leptin was low at <0.6 ng/ml (0.7 to 5.3 ng/ml normal range) as was adiponectin at 3 mcg/ml (normal range 4-20 mcg/ml). His CK, cholesterol, triglycerides, LDL, homocysteine, apolipoprotein B, and lipoprotein A were normal. His HDL was slightly low. A skin biopsy was sent for electron microscopy and mitochondrial structure was unremarkable. He has no history of alcohol abuse.

Patient 2 had a history of 'bowed limbs' requiring casting in infancy, however was very healthy and physically active throughout early and mid-adulthood. He noted a change in his physique around age 45 with evidence of lipomatosis by his early 50s. He has a history of multiple encapsulated cervical and thoracic lipomas, with frequent surgical interventions. He has an approximately 15 year history of progressive neuropathy, and normal HbA1C. He had elevated lactate (3.0 mmol/L, upper limit of normal 2.2 mmol/L). He also had an elevated CK as high as 550 U/L. He had a muscle biopsy as part of his work up which reported

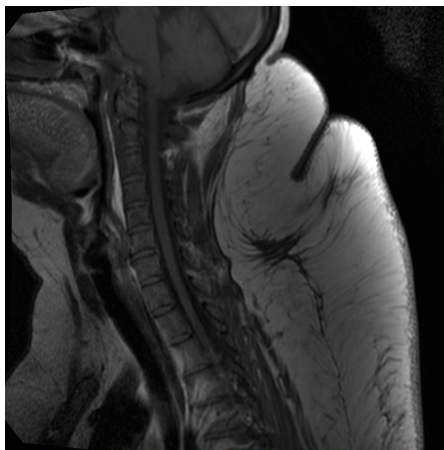
chronic denervation and frequent highly atrophic cd56 and esterase positive myofibers and marked myofiber grouping, rare vacuoles, increased internalized nuclei, and rare RRFs.

The brothers have two unaffected siblings. The parents were of Irish descent and are 5th degree cousins. Both parents are deceased, and neither was diagnosed with CMT or MSL. Both Patient 1 and 2 have children in their early 20's, none of whom have apparent MSL or gait abnormalities.

5.4.2 Patient 3

This patient has been previously described as individual 3 in Australian CMT40 family described in Table 2 [6]. She presented with bilateral club feet at birth and had corrective surgery at 7 years of age, which was unsuccessful. Hearing loss was also noted in early childhood. At the age of 12 years she has muscle wasting and weakness in her legs and she could not feel heat or pain in her feet. At 34 years of age she was fitted with hinged AFOs and at 48 years of age she required a cane to ambulate and a wheel chair for longer distances. She developed pronounced cervical and thoracic lipomatosis in her late 20's, as well as fatty nodules on her forearms and chest (Fig 5.1). She had progressive muscle wasting of her lower extremities with distal weakness (1/5 at the ankles) and deltoid weakness at 4/5 by age 38 years. The distal and lower limb reflexes were absent and at age 38 years she had no detectable sensory action potentials, no lower limb motor responses and a median conduction velocity of 44 m/sec with a latency of 3.3 msec and distal latency of 3.3msec. MRI of the neck showed an accumulation of subcutaneous, unencapsulated fat posterior to the cervical spine folded to a depth of 10.5 cm extending down the lumbar spine with additional significant subcutaneous fat in the thorax, chest, abdomen pelvis and upper thigh (Fig 5.2).

A



B



Fig 5.2: MRI findings in Patient 3. (A) MRI image showing homogeneous unencapsulated cervico-thoracic subcutaneous fat distribution. (B) MRI image showing normal image of the liver, there is no evidence of visceral fat deposition in the patient.

No discrete lipomata were obvious. There was diffuse atrophy and fatty marbling of most of the musculature with some sparing of the iliopsoas muscles. There was a raised blood lactate at 3.1 mmol/L (upper limit of normal 2.1 mmol/L), normal apolipoproteins A1 and B, cholesterol, and triglycerides were normal range. A random blood glucose was 7.2mmol/L. Sanger sequencing identified a homozygous substitution c.2119C>T;(p.R707W) (NM_001127660). Her parents and two daughters all had minor symptoms and signs suggestive of CMT including slow running, recurrent ankle sprains, difficulty heel walking and slight *pes cavus* or flat feet, but had normal neurophysiology and thus were not diagnosed with CMT.

5.4.3 Whole-exome sequencing

Given that the brothers had the same rare disease and there was a remote history of consanguinity, the variants were first filtered for shared rare variants carried in the homozygous state. WES identified a rare homozygous variant shared by both brothers in exon 17 of *MFN2* at c.2119C>T, p.R707W (NM_001127660) that has been reported to be associated with CMT. The gene was in an extended region of homozygosity seen in the brothers that spanned 6.4 Mb in length, consistent with the remote history of consanguinity. This mutation was not present in either of the two unaffected siblings in a homozygous or heterozygous state. No other rare homozygous variants were identified that were shared by the brothers. The mutation was predicted pathogenic by SIFT, Polyphen2, and MutationTaster. The mutation is present in the heterozygous state in the Exac database of 60,000 exomes with a frequency of 0.0003. A broader analysis under different possible modes of inheritance (shared compound heterozygous variants, dominant, or X-linked) was unrevealing.

5.4.4 Functional analysis of p.R707W

We performed cell-based experiments to probe for functional deficits conferred by the R707W mutation and compared results with the severe CMT2A mutation R94W. We observed no difference in mitochondrial morphology in fibroblasts from the index patient compared to normal subjects (Fig S5.1), consistent with the normal morphology in fibroblasts from patients with severe CMT2A [7]. Next, we assessed the capacity of MFN2 R707W to support mitochondrial fusion by monitoring mitochondrial morphology in cells depleted of endogenous MFN1 and MFN2 using RNA interference. Compared to wildtype MFN2, the R707W mutant showed reduced capacity to tubulate mitochondria (Fig 5.3A-B). In addition, mitochondria were more prone to aggregation in cells expressing a MFN2 R707W cDNA tagged with the V5 epitope to facilitate detection (Fig 5.3C). Co-IP experiments in cells expressing V5 and Flag tagged MFN2 revealed a defect of MFN2 R707W to form homo-oligomers, while hetero-oligomer formation with MFN1 was unaffected (Fig 5.3D). When cells expressing V5-tagged MFN2 mutants were subjected to the crosslinking agent BMH, we observed that MFN2 R707W formed smaller oligomeric complexes compared to wildtype MFN2 (Fig 5.3E).

5.5 Discussion

We have identified a recurrent homozygous *MFN2* mutation in patients from two unrelated families with MSL and neuropathy, thereby identifying a second disease gene responsible for this rare condition. Heterozygous mutations in the *MFN2* gene cause CMT2A and the p.R707W is a recognized CMT2A mutation [6]; lipomatosis appears to be unique to individuals carrying this mutation in a homozygous state. There are three siblings described

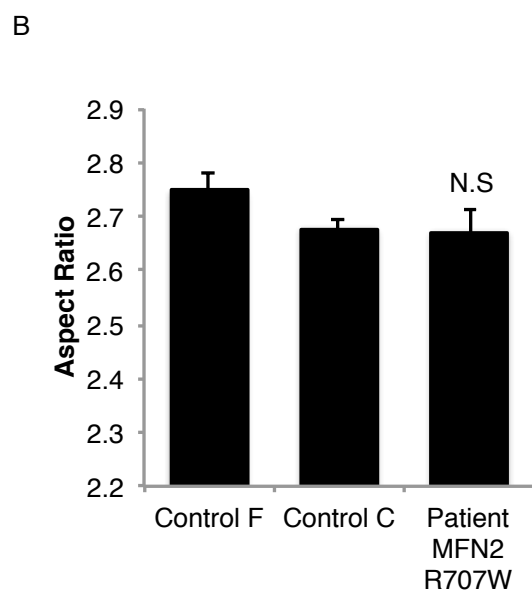
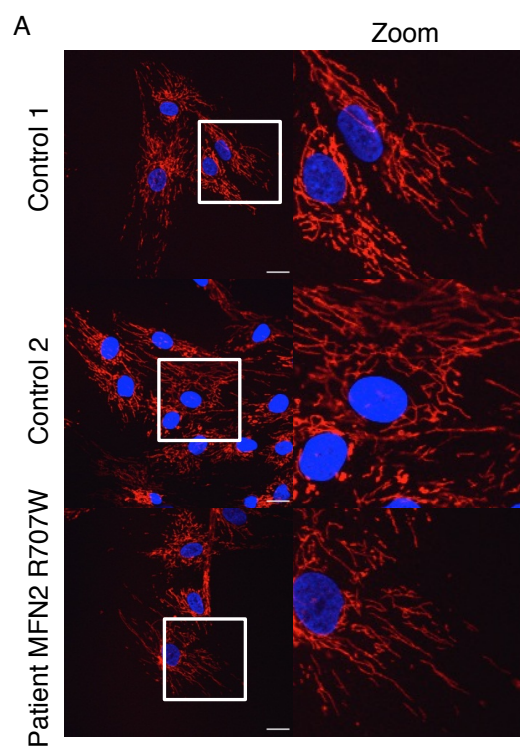


Fig S5.1: Normal mitochondrial morphology in skin fibroblasts from MSL patient with MFN2 R707W mutation. (A) Fluorescence micrographs of fixed skin fibroblasts immunostained with anti TOMM20 (red) and counterstained with Hoechst (blue). Representative micrographs of two control fibroblast cell lines and fibroblast from the MSL patient were shown. Right panels showed zoomed images from the insets drawn on the left panels. Scale bar = 20 μ M. (B) Bar plot showing quantitation of mitochondrial length as assessed by aspect ratio measurements. N.S. = Not significant

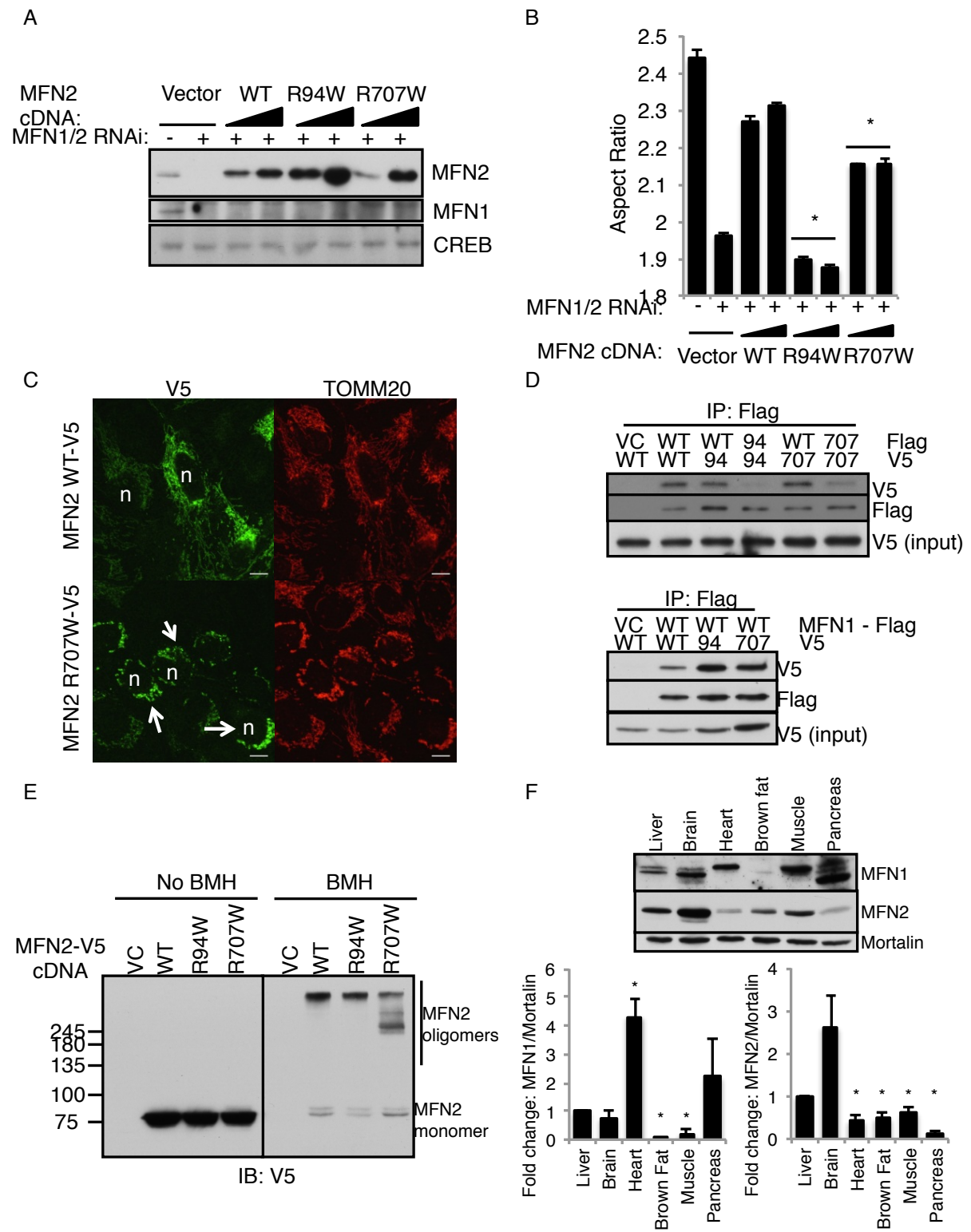


Fig 5.3: Functional Studies. (A) Western blot showing levels of MFN2 and MFN1 proteins in the experiment from 3A. CREB is used as a loading control. (B) Bar plots showing average mitochondrial length of U2OS cells treated by the indicated RNAi and expression constructs. Two doses of MFN2 construct were used. * = $p < 0.005$ compared to MFN1/2 RNAi + MFN2 WT condition. (C) Representative fluorescence micrographs of U2OS cells expressing either V5-tagged MFN2 WT or MFN2 R707W immunostained with antibodies against V5 (green) and TOMM20 (red). White arrows indicate cells with perinuclear aggregation of mitochondria in MFN2 R707W-V5 expressing cells. n = nuclei. (D) Western blot of co-immunoprecipitation experiment. Top 3 panels: U2OS cells expressing various MFN2 constructs tagged with either Flag or V5 was subjected to anti-Flag agarose. Levels of co-immunoprecipitated MFN2-V5 were analyzed by Western blotting. Bottom 3 panels: same as top panels but levels of MFN2-V5 was analyzed in MFN1-Flag immunoprecipitates. 94 and 707 refer to MFN2 R94W and MFN2 R707W, respectively. (E) Western blot of lysates from cells expressing various MFN2-V5 constructs and treated with BMH. Two exposures of film are shown. (F) Western blot showing relative protein levels of MFN1 and MFN2 in various mouse tissues. Mortalin protein is used as a mitochondrial loading control. Bottom two panels: Bar plot of normalized MFN1 and MFN2 in various tissues relative to those found in liver. N = 3 technical replicates. * = $p < 0.05$. VC: Vector Control, WT: wild type, BMH: bismaleimido-hexane, IP: immunoprecipitation, IB: immunoblot

in the literature who are compound heterozygous carriers of the p.R707W and a p.G108R mutation associated with CMT2A and ‘lipodystrophy’ [8], although it is unclear whether MSL was a feature. Patients with compound heterozygote mutations in *MFN2*, that do not include the p.R707W mutation, have also been reported [6] without MSL or lipodystrophy. Thus, it appears that the p.R707W mutation, at least in the homozygous recessive form, is specific to the etiology of the MSL phenotype. Obligate carriers of the p.R707W mutation in these two families had not been diagnosed with CMT2, despite clinical assessment of the children of Patient 3, which may be secondary to a mild presentation, later onset, or other modifying risk factors.

The identification of *MFN2* as a MSL disease gene contributes to our understanding of the role of mitochondria in brown fat metabolism. *MFN2* encodes a GTPase of the dynamin family that is localized to the mitochondrial outer membrane, and mediates the homotypic interaction and fusion of apposed mitochondria [9]. The p.R707W alteration is found within the heptad repeat 2 (HR2) domain located at the C-terminus of *MFN2* which is thought to permit homotypic interactions as well as heterotypic binding to *MFN1*. *MFN2* homozygous p.R707W mutant cells demonstrate a reduced ability to form homotypic interactions in vitro and tubulate mitochondria. The presence of *MFN1* has been proposed to mask the consequences of *MFN2* disease alleles in most tissues [10]. Studies looking at the relative amounts of *MFN1* and *MFN2* protein in various tissues demonstrated that while *MFN1* is enriched in the heart, in agreement with previous data [11], it was almost undetectable in brown fat (Fig 5.3F), a tissue implicated in the pathogenesis of MSL. These data are in agreement with the notion that deleterious mutations in *MFN2* can lead to mitochondrial dysfunction in brown fat [12], and support *MFN2* as a novel gene causing MSL.

5.6 Materials and Methods

5.6.1 Whole Exome Sequencing

Patient 1 and 2 underwent whole exome sequencing as part of the Care4Rare Canada Consortium with the objective to identify novel causes of rare disease. Institutional research ethics board (Children's Hospital of Eastern Ontario) approval of the study design was obtained, and informed consent was obtained. DNA was extracted from blood according to standard protocols. Targeted enrichment was performed using the Agilent SureSelect 50Mb (V5) All Exon Kit. The Illumina HiSeq 2000 was used to sequence the captured product and three samples were run per lane. Coverage of the exome was between 135-140x. WES data analysis was performed using our standard pipeline as previously described [13]. Variants were annotated with the functional type (e.g. nonsense versus a missense), allele frequency data using both an in-house control sample database (filtered to less than 3%) and frequency data from the Exome Variant Server (filtered to less than 1%). Candidate mutations were validated by Sanger sequencing and segregated in the siblings.

5.6.2 Functional analysis of p.R707W

Lentivirus preparation was performed as described previously [14]. Lentiviral vector encoding MFN2 shRNA targeted the following sense sequence: GCAGGTTTACTGCGAGGAAAT. MFN1 siRNA was from Dharmacon (L-010670-01). For lentiviral constructs expressing MFN2 and MFN1, MFN2 and MFN1 cDNA were gateway recombined into pLenti6.3 V5 DEST vectors (Invitrogen). R94W, R707W and MFN2 shRNA-resistant cDNAs were generated by quickchange site-directed mutagenesis. Antibodies to TOMM20 (FL-145) and mortalin were from Santa Cruz. MFN1 antibody was obtained from Abcam (ab104274). MFN2 antibody was from Abnova (H00009927-M03J).

V5 antibody was from Invitrogen. For tissue blots, antibody to MFN1 was from Cell Signaling. Flag (M2)-HRP antibody was from Sigma. Immunofluorescence, confocal microscopy and Co-immunoprecipitation (CO-IP) was performed as previously described [15]. Mitochondrial length measurements (aspect ratio) and BMH (bismaleimido-hexane) crosslinking experiments were performed as described previously [16]. Tissue for blots was harvested from C57BL/6 wild type mice and subjected to homogenization using TissueLyser (Qiagen). Insoluble material was removed by centrifugation prior to SDS-PAGE and Western blotting.

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5.8 Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interests.

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Chapter 6

General Discussion

Mitochondrial dysfunction contributes to the pathogenesis of a growing number of acquired and inherited human diseases. As mitochondria are the main sites of ATP generation, mitochondrial diseases usually affect energetically demanding tissues, such as neurons, muscles, and pancreatic beta cells. A consequence of high substrate feed into mitochondria is the premature slippage of electrons, leading to the generation of damaging free radicals. Therefore this underscores the importance of elucidating factors and pathways that preserve mitochondrial health and integrity. Mitochondrial dynamics, and clearance of damaged proteins and organelles are considered critical aspects of mitochondrial quality control. Mitochondria undergo fusion to complement damaged genomes and proteins. Mitochondria undergo fission as a prerequisite for mitochondrial delivery (for example: to axon terminals) and mitophagy of terminally-damaged mitochondria. In this work, we provided evidence of two novel players critically involved in mitochondrial quality control. We showed that TID1 prevents mitochondrial protein misfolding, regulates $\Delta\psi_m$, and maintains mitochondrial genomes, whereas ROMO1 maintains mitochondrial fusion and has additional roles in metabolism.

6.1 Genome-wide RNAi screen

In this thesis, I developed and executed a genome-wide RNA interference screen with the aim to identify novel regulators of mitochondrial dynamics. To our knowledge, we are the first group to use an automated detection and algorithmic scoring of mitochondrial length. This allowed us to overcome biases of manual morphologic scoring. Average mitochondrial length in ~55,000 sample wells (18,255 genes X triplicates) were screened. 8 fields per well were imaged using automated Cellomics Vti microscope equipped with a 40X objective. To report on the aspect ratio, we used the MEAN_ObjectEqEllipseLWRCh1 metric within the Morphology Bioapplication provided with the Cellomics vHCS. Robust Z-scores were used to assess the change in

mitochondrial length from the median of the sample population. The top 200 candidate genes from fragmentation and elongation end were visually assessed to confirm changes in mitochondrial morphology. Secondary screens were performed using deconvoluted (unpooled) siRNA reagents. Candidate genes in which three or more siRNAs producing the same phenotype were considered top candidate genes. Lentivirus expressing siRNA-resistant cDNA were generated and rescue experiments were performed. The genes *ROMO1*, *TID1*, and *ATPIF1* were identified using this method.

We optimized the detection of mitochondrial fluorescence signal and rejected dense, perinuclear mitochondria and background fluorescence in order to further separate the morphologic phenotypes. The mitochondrial length score, which we have termed “aspect ratio”, is based on mitochondrial signal from over 80 cells (8 fields per well X ~10 cells per field). When experiments are performed in quadruplet wells, the aspect ratio score highly correlates with the actual mitochondrial length and is highly reproducible as evidenced by the small error bars from our published work [1].

Despite our attempts to maximize the quality of fluorescence images generated by the high-throughput protocol, we were unable to achieve a positive Z' factor (i.e: >0), suggesting that our screen may generate a large number of candidates showing mild phenotypes, which necessitate downstream validation efforts. Indeed, this was the case, and manual inspection of hundreds of images from both elongation and fragmentation end of the spectrum of the candidate list was required. Similarly, our screen to identify genes required for mitochondrial recruitment of PARK2 also required manual inspection of large numbers of images.

We have spent considerable time ruling out false positives from true positives. Although RNA interference has proven invaluable in elucidating function of genes for over a decade, unintended silencing of genes (RNAi off-target effects) continues to plague experiments

using RNA interference. Practically, a RNA interference screen at a genome-wide level will invariably generate a large number of false positives due to off-targeting of siRNAs [2, 3]. A phenotype that can be achieved by two or more siRNAs targeting different regions of the transcript is indicative of a true positive, as we have observed for *TIDI*, *ROMO1*, and *ATPIF1*. The gold standard for hit validation is to “rescue” the phenotype by ectopic introduction of a siRNA-resistant cDNA.

With advancing technology, we strived to continue optimizing and updating our methods for automated measurements of mitochondrial length. Our first genome screen was performed using MitoTracker Red (MTR), a derivative of X-rosamine, which loads into mitochondria based on $\Delta\psi_m$. This first genome screen identified a gene called *TIDI*, where silencing of this gene led to punctate MTR staining (hot spots) suggestive of mitochondrial fragmentation. Unexpectedly, loss of *TIDI* instead affected the distribution of $\Delta\psi_m$ without grossly affecting mitochondrial morphology [4]. We have subsequently discovered that immunostaining using TOMM20 antibody yielded better separation of fused and fragmented mitochondria and allowed us to overcome the dependence on $\Delta\psi_m$ -loading. As such, we used this updated protocol and repeated the genome screen, which led to the discovery of *ROMO1* as a regulator of mitochondrial fusion [1]. Finally, we have performed another genome screen using the Opera confocal microscope, which allowed us to generate fluorescence images of even higher quality. As a result of this third screen, we have discovered and are currently validating a role of the novel gene *MTX2* in regulating mitochondrial dynamics.

6.2 Discovery and characterization of *TIDI*

Several groups have suggested a role for *TIDI* in modulating various cytosolic pathways. It must be noted that many of these systems use exogenously expressed proteins, which often accumulate to non-physiological levels [5-8]. Theoretically, it is possible that newly

synthesized TID1 proteins may briefly function as DNAJ proteins for cytosolic HSP70s in transit to mitochondria [9]. Due to the presence of a N-terminus mitochondria-targeting presequence [10], we and others have observed that endogenous TID1 almost exclusively accumulate in mitochondria [4, 10].

J domain-containing proteins function as co-factors and activate the low-basal ATPase activities of HSP70 proteins. There are 41 putative DNAJ proteins, 4 of which are mitochondria-localized [11]. Multiple J-domain cofactors may function with a single HSP70, thereby providing it with functional diversity. This is exemplified in the case of mortalin/HSPA9, the only mitochondrial HSP70 identified. Cell-free evidence points to a role of TID1 in refolding and disaggregating proteins [12]. However until now, specific substrates have not been identified. It is possible that TID1 could be responsible in folding all aggregated or misfolded mitochondrial proteins. Our data provided convincing evidence that TID1 interacted and prevented the aggregation of complex I subunits, suggesting that complex I subunits are TID1-client proteins. Silencing of *TID1* led to formation of discrete foci characterized by high $\Delta\psi_m$. The mechanism of formation of these “hot spots” is not fully elucidated, but requires a functional respiratory chain and colocalizes with the complex I subunit NDUFS3.

Mutation in *Mdj1p*, the yeast homolog of *TID1*, led to a loss of mitochondrial DNA. Heart-specific *Tid1* knockout also showed a loss of mitochondrial DNA [13]. However, the loss of mitochondrial genome can be caused by a variety of factors such as chronic inhibition of mitochondrial protein import, loss of mitochondrial dynamics, or general mitochondrial dysfunction. It is therefore unclear whether *TID1* is directly involved in maintaining mitochondrial DNA maintenance. To this end, we performed biochemical analysis and RNA interference experiments. We showed that detergent-soluble fraction of TID1 can be

precipitated using spermidine, a DNA-precipitating agent, suggesting a significant fraction of TID1 is associated with mitochondrial DNA. Acute *TID1*-silencing led to loss of mitochondrial DNA, consistent with the idea that TID1 maintains mitochondrial DNA integrity.

The chronic inhibition of ATP synthase by oligomycin treatment depleted the mitochondrial pool of ATP, faithfully phenocopying TID1 silencing. Invoking the nucleotide-dependent mechanism of DNAJ-HSP70 protein-chaperone binding and release cycle, we hypothesized that without ATP, misfolded complex I proteins stably associated with TID1-mortalin-ADP complex. We also showed that oligomycin treatment substantially increased the protein levels of TID1, suggestive of a response to an accumulation of misfolded protein. The migration of detergent-soluble TID1 to detergent-insoluble TID1 to mediate complex refolding represents an elegant mechanism that explains a dual role of *TID1* in preventing complex I aggregates and maintaining DNA integrity.

6.3 Role of *ROMO1* in regulating Spare Respiratory Capacity

Our genome-wide screen for novel regulators of mitochondrial dynamics led to the discovery of *ROMO1*. Matthew Norton in our lab showed that ROMO1 is required for OPA1 activity and mitochondrial fusion. Furthermore, he demonstrated that *ROMO1*-silencing led to a loss of spare respiratory capacity (SRC).

Several lines of evidence point to an inhibition of substrate oxidation as a cause of the loss of SRC. First, providing excess substrates restored SRC. Second, electron transport chain (ETC) integrity is unaffected by *ROMO1*-silencing. Third, *ROMO1*-silencing led to a high dependence on pyruvate oxidation in maintaining basal respiration, suggesting an inability to switch to another substrate to maintain basal respiration. Alternatively, *ROMO1*-silencing decreases pyruvate oxidation to a point where SRC could not be maintained, but still

sufficient to drive basal respiration. Further inhibition of pyruvate oxidation in *ROMO1*-silenced cells using UK5099 completely collapsed basal respiration. Future metabolic flux experiments using heavy glucose labeling will help identify which metabolic pathways are affected in cells lacking *ROMO1*. Given the importance of SRC in regulating multiple facets of human (patho)physiology, we propose that identifying the mechanisms regulating SRC can lead to novel therapeutic targets.

The physiologic role of *ROMO1* is unclear. We observed that whole-body *Romo1* knockout mouse is embryonic lethal (unpublished observations), which precluded an analysis of the physiologic role of *Romo1* in the developed animal. Our laboratory is currently generating inducible brain, heart, and pancreatic beta cells knockouts by crossing mice with a floxed *Romo1* allele with strains carrying the *Camk2a-Cre^{Tam}*, *MHC-Mer/Cre/Mer*, and *Pdx1-CreER^{tam}* transgene, respectively. These tissues were chosen because they are energetically demanding, and their physiological functions require functioning mitochondria, thus increasing the likelihood of observing physiologic phenotypes when *ROMO1* is ablated.

6.4 Homozygous mutations in *MFN2* cause Multiple Symmetric Lipomatosis with Neuropathy

In collaboration with Dr. Sarah Sawyer and Dr. Kym Boycott, we showed that homozygous mutations in *MFN2* c.2119C>T: pR707W cause multiple symmetric lipomatosis (MSL). Two brothers, who developed progressive lipomas and peripheral neuropathies by their 50s, both possess this homozygous mutation, which was not present in their unaffected siblings. An unrelated patient in an Australian patient series with the same homozygous mutation also presented with CMT-type symptoms with cervical and thoracic lipomatosis. These data from two unrelated families provide strong evidence that *MFN2* c.2119C>T: pR707W is causal for MSL. Immunoprecipitation and crosslinking experiments using mutant *MFN2* expression

constructs clearly showed a defect in MFN2 complex formation. These data are consistent with the notion that the Arg707 residue, which resides in the HR2 domain, is critical for homo- and hetero-oligomers. We observed that MFN1 is present at low levels in brown fat, a tissue implicated in MSL. This is in agreement with the notion that the effects of *MFN2* mutations manifest in tissues with low MFN1 levels [14]. Due to the unavailability of a human brown fat cell line, we were unable to test the effects of MFN2 R707W in this tissue. Patient lipomas from liposuction were available, but practical constraints in working with primary fatty tissue and a lack of control samples ruled out analysis of mitochondrial morphology with these samples. However, it would be necessary to confirm, in patient lipomas, the presence of uncoupling protein 1, a marker of brown adipocytes. This would greatly strengthen the assertion that mitochondrial dynamics dysfunction specifically in brown fat leads to MSL.

6.5 Major Limitations

Most of the data presented in this studies were derived from experiments using transformed cell lines (U2OS and HeLa). The main reasons for the use of transformed cell lines were the ease of overexpression and downregulation of gene expression, ability to obtain a large amount of protein material for biochemistry, and the mitigation of biologic variability that would be encountered if primary cells derived from different animals were used. The use of transformed cell line allowed us to uncover, what we confidently believe to be, the true functions of *TIDI*: disaggregate complex I and maintain mitochondrial DNA fidelity. This would otherwise have been difficult, if not, impossible, if we had used primary cells or an animal knockout approach. The main disadvantage of using transformed cell lines is that transformed cell lines often lose their ability to appropriately respond to physiological relevant signals compared to the response in non-transformed cells. Transformed cell lines

may have altered response to catecholamines (eg: transformed brown adipocytes) and glucose-stimulated insulin secretion (eg: HIT, MIN6 cell lines). We are currently developing a genome-wide screening platform using primary cells. This will allow us to uncover novel genes that respond in a physiological signal-dependent manner and modulate relevant downstream biology.

In chapter 5, we provided data that strongly suggest a mitochondrial etiology in the development of MSL. We have also implicated mitochondrial dynamics in the pathogenesis of MSL. This is in agreement with the recently discovered role of mitochondrial dynamics in brown fat metabolism [15]. The authors showed that mitochondrial fragmentation augments energy dissipation by further uncoupling ATP synthesis from fatty-acid derived substrate feed. In this thesis, the connection between brown fat and MSL remains correlative. Further research using MSL patient-derived lipomas is needed to establish a brown-adipocyte (BAT) cause of MSL. For example, positive UCP1 expression in patient lipomas will provide strong evidence of a BAT etiology. However, imaging and biochemical experiments using lipomas remain challenging due to the high fat content of these samples.

6.6 Concluding Statement

This thesis demonstrated that genome-wide loss of function screens can be used to identify novel regulators of mitochondrial quality control. We screened for genes required for regulation of mitochondrial dynamics and uncovered *TID1* and *ROMO1*. We uncovered a novel TID1-mediated stress response to ATP synthase inhibition. We demonstrated dual roles of *TID1* in maintaining mitochondrial DNA integrity and preventing complex I misfolding, two processes critical for mitochondrial health. In addition, we showed that *ROMO1* played a role in mitochondrial fusion and maintenance of spare respiratory capacity.

Our discovery and characterization of *ROMO1* now leads us to mouse studies, in which, we will assess the physiological impact of *Romo1* ablation in various energetically demanding tissues.

The last part of this thesis demonstrated the importance of mitochondrial dynamics in human diseases. We now include MSL, in addition to CMT, as diseases associated with *MFN2* mutations. Our work sheds light on the pathogenesis of MSL by showing how a specific molecular defect in a mitochondrial protein led to the development of a rare lipid disorder.

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Appendix 1

Genome-wide RNAi Screen Identifies ATPase inhibitory factor 1 (ATPIF1) as Essential for PARK2 Recruitment and Mitophagy

Preface

Appendix 1 contains a published paper on *ATPIF1*, a gene that we have discovered through a genome screen for genes that regulate the uncoupler-mediated mitochondrial translocation of PARK2/Parkin. We showed that silencing *ATPIF1*, an endogenous inhibitor factor of the ATP synthase, prevented uncoupler-mediated mitochondrial recruitment of PARK2. We showed that ATPIF1 mediated the loss of $\Delta\psi_m$, leading to the activation of PINK1-PARK2 and subsequent mitophagy.

Author list

Valerie Lefebvre, Qiujiang Du, Stephen Baird, Andy Cheuk-Him Ng, Mirna Nascimento, Michelangelo Campanella, Heidi M. McBride, Robert A. Screatton

Author contribution

VL performed the genome screen that led to the identification of *ATPIF1* and performed most of the experiments in this manuscript. QD performed the FACS analyses to measure mitophagy. SB helped with the quantitation of fluorescence images. AC-HN designed and optimized the initial genome screening protocol. RAS helped with experimental design, supervised the study and wrote the manuscript.

Abstract

Mitochondrial dysfunction is a hallmark of aging and numerous human diseases, including Parkinson disease (PD). Multiple homeostatic mechanisms exist to ensure mitochondrial integrity, including the selective autophagic program mitophagy, that is activated during starvation or in response to mitochondrial dysfunction. Following prolonged loss of potential across the inner mitochondrial membrane ($\Delta\Psi$), PTEN induced putative kinase 1 (PINK1) and the E3-ubiquitin ligase PARK2 work in the same pathway to trigger mitophagy of dysfunctional mitochondria. Mutations in PINK1 and PARK2, as well as PARK7/DJ-1, underlie autosomal recessive Parkinsonism and impair mitochondrial function and morphology. In a genome-wide RNAi screen searching for genes that are required for PARK2 translocation to the mitochondria, we identified ATPase inhibitory factor 1 (ATPIF1/IF1) as essential for PARK2 recruitment and mitophagy in cultured cells. During uncoupling, ATPIF1 promotes collapse of $\Delta\Psi$ and activation of the PINK-PARK2 mitophagy pathway by blocking the ATPase activity of the F_1F_0 ATP synthase. Restoration of ATPIF1 in Rho0 cells, which lack mtDNA and a functional electron transport chain, lowers $\Delta\Psi$ and triggers PARK2 recruitment. Our findings identify ATPIF1 and the ATP synthase as novel components of the PINK1-PARK2 mitophagy pathway and provide genetic evidence that loss of DY is an essential trigger for mitophagy.

Introduction

Mitochondria are essential for cellular metabolism, including the generation of ATP from nutrients via oxidative phosphorylation (“respiration”), metabolite interconversion, calcium ion and amino acid flux, and also govern cell fate in response to environmental changes. Mitochondrial dysfunction is associated with both aging and human diseases, including degenerative neuromuscular disorders, diabetic or metabolic conditions, cancer and Parkinson disease,[1] as well as mitochondrial myopathies.[2-4] There is now evidence that multiple programs have evolved to ensure mitochondrial integrity through homeostatic mechanisms, including intramitochondrial proteases[5] and the ubiquitin-proteasome system.[6] The selective autophagic program of mitochondrial degradation, mitophagy, serves to remove mitochondria during starvation, as well as mitochondria that fail to restore potential across the inner mitochondrial membrane (IMM).[7-9] A pathway has been identified in metazoans featuring PINK1 and PARK2 serves to trigger mitophagy in response to prolonged loss of potential across the IMM, a key mechanism in mitochondrial quality control.[10-15] Mutations in PINK1, PARK2 and PARK7 underlie autosomal recessive Parkinsonism and cause alterations in mitochondrial function and morphology.[16-19]

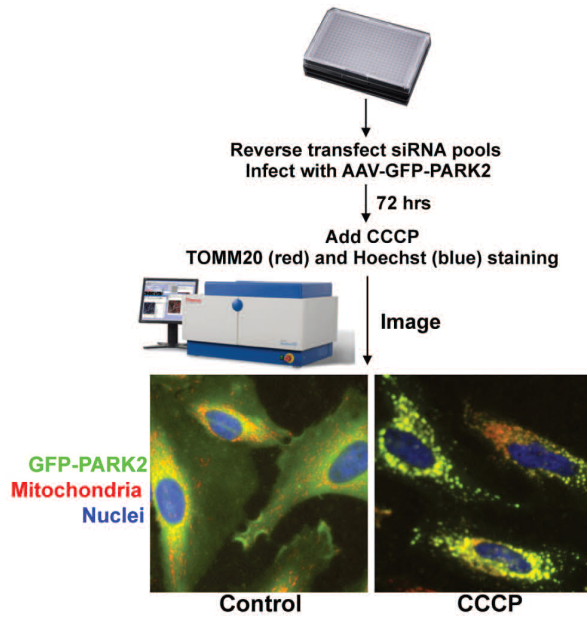
How PINK1 signals to PARK2 and the physiological stimulus required for PINK1 activation is unclear. To identify additional components of this pathway, we performed a genome-wide RNAi screen in HeLa cells and report that *ATPIF1* is an essential gene for PARK2 recruitment and for mitophagy. We provided evidence that the ATPase activity of mitochondrial F_1F_o -ATP synthase (hereafter referred to as ATP synthase) is sufficient to maintain $\Delta\Psi$ in CCCP-treated cells at the expense of cellular ATP, thus preventing PARK2 recruitment and mitophagy. ATPIF1 blocks ATPase function, thus promoting collapse of $\Delta\Psi$

and activation of the PINK-PARK2 mitophagy pathway. These results demonstrate that the ATPIF1-ATPase complex is an essential upstream regulator of mitophagy.

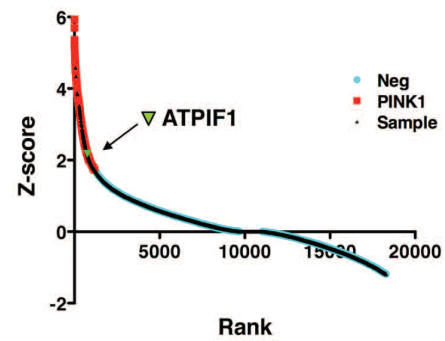
Results

HeLa cells expressing GFP-PARK2 were arrayed in 384-well plates and transfected with the human genome-wide siRNA library from Dharmacon. Three days later, cells were treated for 1 h with the mitochondrial proton gradient uncoupler CCCP (Fig 1A), fixed, and stained for mitochondria and nuclei. The GFP-PARK2 signal was monitored by automated microscopy. We used an algorithm that identifies GFP-PARK2 signal colocalized with the outer mitochondria membrane marker, TOMM20 to distinguish control siRNAs (high colocalization with TOMM20) from hit siRNAs (low colocalization with TOMM20). ATPIF1 was identified as a hit with a z-score that fell within the positive controls (Fig 1B, Data S1). Indeed, three out of four siRNA duplexes targeting ATPIF1 completely prevented PARK2 recruitment, scoring at the level of the PINK1 internal control (Fig 1C-D).[12-14] Western blot analysis demonstrated efficient knockdown of ATPIF1 protein with the three effective siRNAs (Fig 1E). To confirm these data, we infected HeLa cells with a lentivirus expressing an shRNA targeting a different region of the *ATPIF1* mRNA and again observed inhibition of PARK2 recruitment following CCCP treatment (Fig S1). Importantly, a lentivirus expressing a V5-tagged siRNA-resistant *ATPIF1* cDNA completely restored PARK2 recruitment in ATPIF1 knockdown cells treated with CCCP (Fig 1F-G). Furthermore, mutation of two key residues required for binding to the F₁ domain of ATP synthase, E55 and Y58, prevented restoration of recruitment,[20] demonstrating that binding of ATPIF1 to the F₁-ATPase is essential for PARK2 recruitment (Fig S2). Knockdown of the

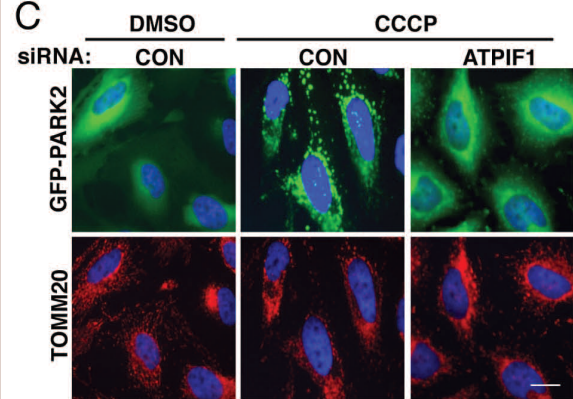
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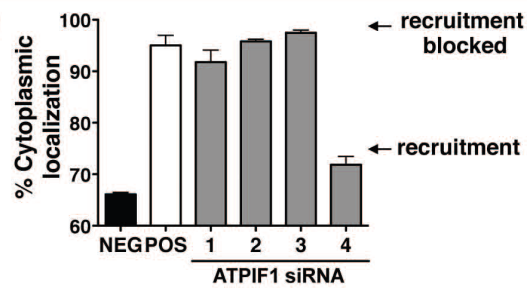
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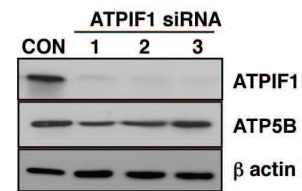
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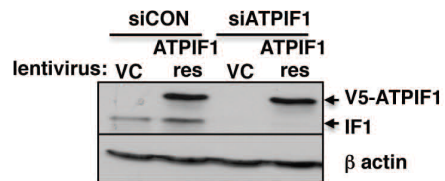
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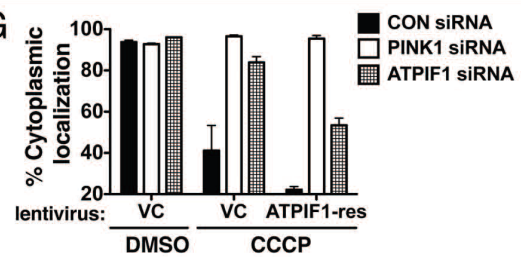


Fig 1: ATPIF1 is required for PARK2 recruitment following loss of $\Delta\Psi$. (A) Layout of genome-wide RNAi screen conditions. HeLa cells were simultaneously transfected with arrayed pools of 4 siRNA in 384-well clear-bottom microplates and infected with AAV-PARK2-GFP in a high-throughput format. Following knockdown, cells were treated with the mitochondrial uncoupler CCCP (5 μ M) for 1 h to induce GFP-PARK2 recruitment to mitochondria. (B) Q-Q plot showing Z-score for siRNA pools (ranks) in the genome screen. Scrambled nonspecific siRNA negative (blue squares) and PINK1 siRNA positive (red squares) controls are shown. The ATPIF1 siRNA pool is indicated by the green triangle. (C) Fluorescence micrographs showing failure of GFP-PARK2 recruitment to mitochondria following CCC P treatment in ATPIF1 knockdown cells. Outer mitochondrial membrane marker TOMM20 staining (red) and nuclei (blue) are shown. DMSO as the vehicle control for CCC P treatment is indicated. Scale bar: 25 μ m. (D) 3 out of 4 siRNAs from the siRNA SmartPool targeting ATPIF1 block PARK2 recruitment. The effect of the PINK1 positive control siRNA is shown. CON, control siRNA. Data are mean $\pm$ SD of 4 independent experiments. (E) Western blot reveals efficient knockdown of ATPIF1 by siRNAs that block PARK2 recruitment. The blot was reprobed with ATPase ATP5B subunit antibody for loading control. (F) Western blot showing expression of ATPIF1 in cells infected with vector control (VC) and lenti-ATPIF1-V5 (IF1-res) virus. (G) Histogram showing restoration of recruitment following reintroduction of siRNA-resistant FLAG-tagged-ATPIF1 cDNA (IF1-RES).

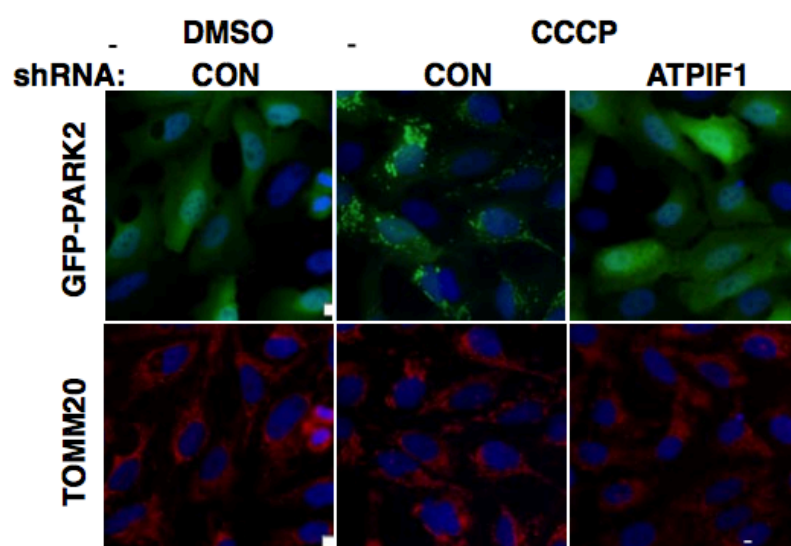


Fig S1: Fluorescence micrographs showing failure of GFP-PARK2 recruitment (top row) in HeLa cells infected with lentivirus encoding an shRNA targeting ATPIF1. Control shRNA (CON), DMSO and CCCP treatment are shown. TOMM20 staining is shown (bottom row). Nuclei are stained with Hoechst. Scale bar, 80 μ m.

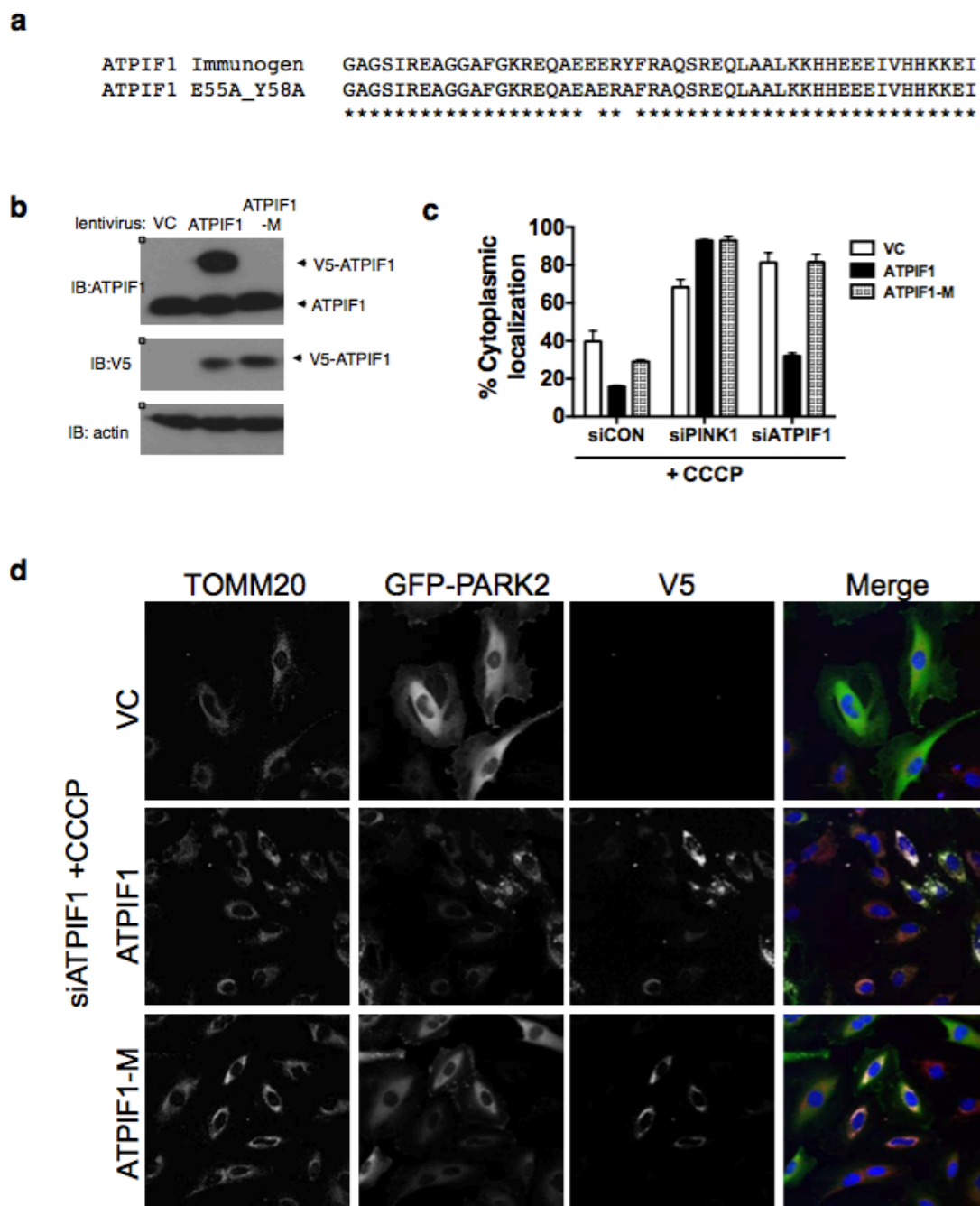


Fig S2: ATPIF1 binding to the F1-ATPase is required for PARK2 recruitment in HeLa cells. (A) Alignment of wild-type ATPIF1 and E55A,Y58A mutant FASTA sequences across the region that served as antigen to generate the commercial ATPIF1 antibody used in this study. (B) Western blotting analysis of V5-tagged WT (ATPIF1) and mutant (ATPIF1-M) protein levels in HeLa cells following 72 h infection. The empty vector control (VC) is shown. (C) Histogram showing the level of PARK2 recruitment in HeLa cells following 72 h period of knockdown of endogenous ATPIF1 and lentiviral reconstitution with siRNA-resistant ATPIF1 or ATPIF1-M constructs. The empty vector control (VC) is shown. (D) Immunofluorescence images of HeLa cells expressing GFP-PARK2 (green), and stained with Hoechst to reveal the nuclei (blue), TOMM20 antibody for the mitochondria (red), and V5 for ATPIF1 localization (white). Cells were subjected to 72 h of knockdown of endogenous ATPIF1 and lentiviral reconstitution with siRNA-resistant ATPIF1 or ATPIF1-M constructs prior to a 1 h treatment with 5 μ M CCCP. The empty vector control (VC) is shown.

kinase LRRK2, another PD associated gene [21] had no effect on blocking PARK2 recruitment (Fig S3), indicating that LRRK2 likely functions in a distinct pathway from PINK1-PARK2. Importantly, these data reveal a unique and highly specific requirement for PINK1 and for no other protein kinase for PARK2 recruitment. Taken together, these data indicate that ATPIF1 is essential for PARK2 recruitment in HeLa cells in response to mitochondrial uncoupling.

When respiration is inhibited, a reduction in $\Delta\Psi$ occurs, and the ATP synthase complex functions as an ATPase, hydrolyzing ATP stores in the matrix to drive restoration of $\Delta\Psi$ across the inner membrane, thus preventing long-term loss of $\Delta\Psi$ in focal regions of the network.[22, 23] In this setting, dimeric ATPIF1 is thought to bridge two ATP synthase complexes[20, 23-25] and prevent ATP hydrolysis and restoration of the proton gradient across the IMM by steric hindrance.[26] Thus, in ATPIF1 knockdown cells following CCCP treatment, the ATPase would theoretically run unfettered to restore $\Delta\Psi$. To test this, we treated control and ATPIF1 knockdown cells with CCCP for 1 h, then incubated them for 30 min with MitoTracker Red (MTR), a potentiometric dye that selectively loads into mitochondria with intact $\Delta\Psi$. As expected, CCCP-treated cells were negative for MTR staining, but ATPIF1 knockdown cells incorporated MTR, indicating they maintained $\Delta\Psi$ (Fig. 2A).[27] TMRE staining revealed that ATPIF1 knockdown cells retained significant (40%) membrane potential following CCCP treatment, an effect that could be blocked by co-treatment with oligomycin, which inhibits both ATP synthesis and ATPase activities of the ATP synthase (Fig 2B). We confirmed that plasma membrane potential was unchanged in ATPIF1 knockdown cells, indicating that TMRE loading was unaffected by ATPIF1 status (data not shown). If loss of $\Delta\Psi$ persists, the ATPase can deplete the cellular

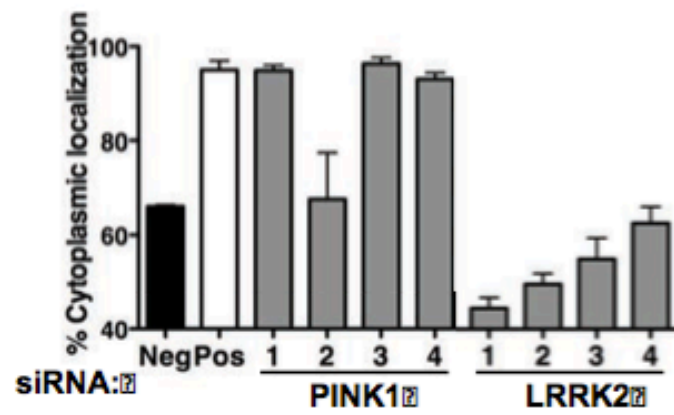
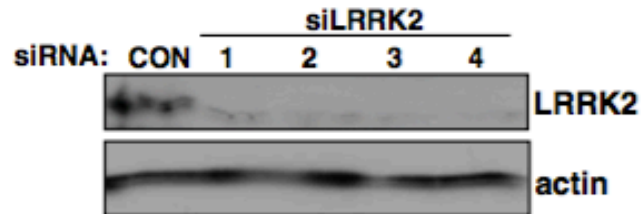
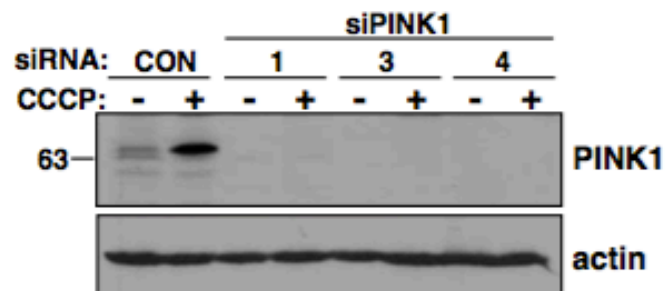
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Fig S3: LRRK2 is dispensable for PARK2 recruitment. (A) Histogram showing quantification of GFP-PARK2 recruitment to mitochondria. Effects of negative control (Neg) as well as individual siRNAs from the RNA pool targeting positive control PINK1 (Pos) and LRRK2 are shown. Whereas all 4 siRNAs targeting LRRK2 had no effect on PARK2 recruitment, 3 out of 4 PINK1 siRNAs blocked recruitment completely. (B) Western blot showing knockdown of endogenous LRRK2 with the 4 functional siRNAs. Actin loading control is shown. (C) Western blot showing knockdown of endogenous PINK1 with the 3 functional siRNAs, with or without CCCP treatment.

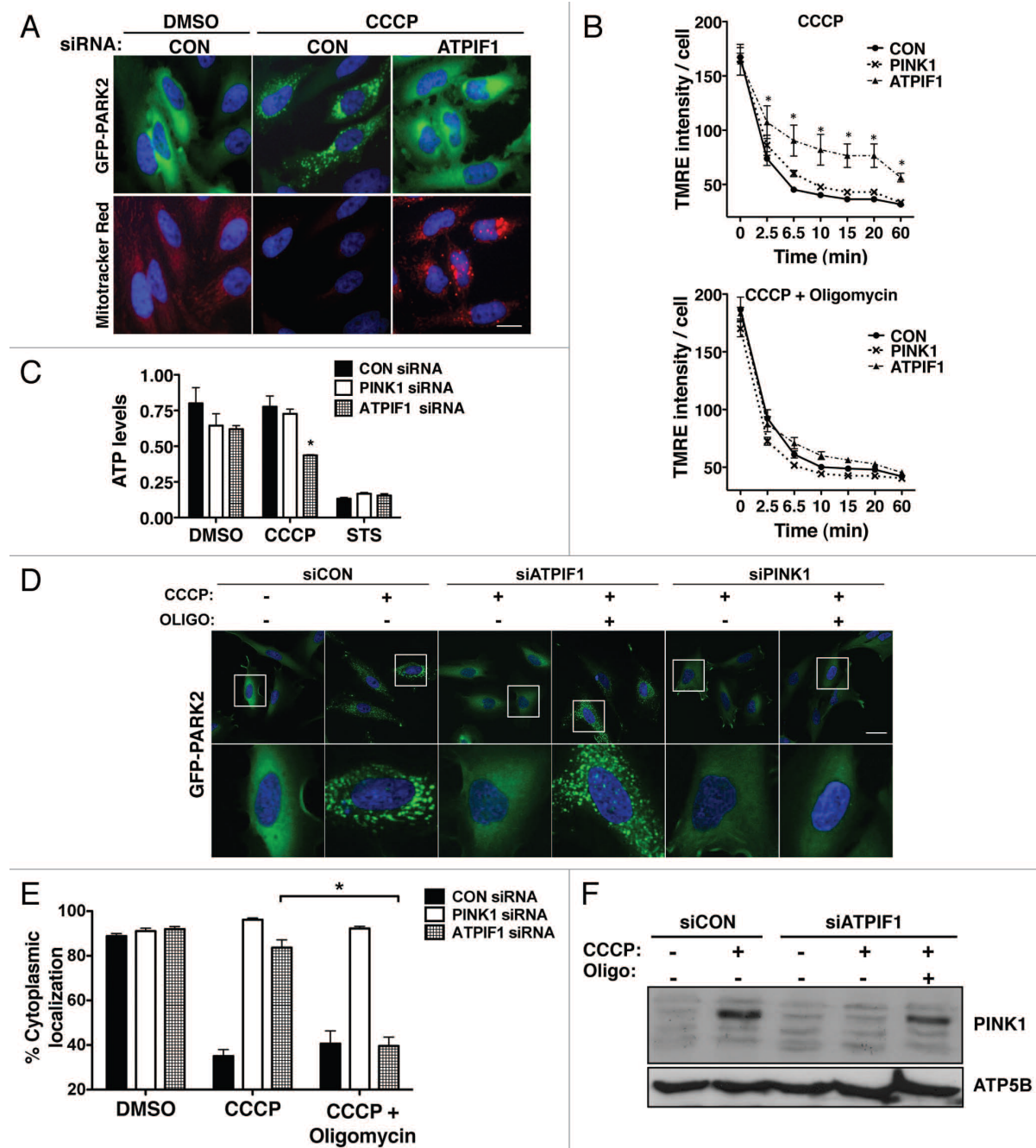


Fig 2: ATPIF1 regulation of PARK2 recruitment by regulating the mitochondrial F1Fo-ATPase upstream of PINK1 activation. (A) Fluorescence micrographs showing loss of mitochondrial IMM potential ($\Delta\Psi$) in CCC P-treated cells as revealed by MitoTracker Red staining. Knockdown with control (CON) or ATPIF1 siRNAs in DMSO vehicle or CCC P is shown. Scale bar: 50 μm . (B) TMRE fluorescence intensity following CCCP (top) or CCCP + oligomycin (bottom) treatment of control, ATPIF1 or PINK1 knockdown cells. Data are mean $\pm$ SD of triplicate measurements of 1 experiment that is representative of 3 independent experiments. * $P < 0.05$, paired t-test. (C) ATP levels expressed as luminescence unit/cell in HeLa cells following 3 h CCCP treatment. Effect of knockdown of PINK1 or ATPIF1, as well as the cytotoxic drug staurosporine (STS) is shown. Data are mean $\pm$ SD of triplicate measurements that is representative of 2 independent experiments. * $P < 0.05$, paired t-test. (D) Fluorescence micrographs showing restoration of recruitment in ATPIF1 knockdown cells following inhibition of F1Fo ATP synthase with oligomycin (OLIGO). Effect of control, PINK1 and ATPIF1 siRNAs is shown. Scale bar: 50 μm . Inset: 4 $\times$ magnification of white box. (E) Histogram showing effect of CCC P and oligomycin treatment in control, PINK1 and ATPIF1 knockdown cells shown in (D). Data are mean $\pm$ SD of 2 independent experiments. * $P < 0.002$, paired t-test. (F) Western blot showing PINK1 levels in control and ATPIF1 knockdown cells treated with CCCP. Oligomycin treatment is indicated.

ATP pool, exacerbating both cell damage and death associated with oxidative stress.[28-30] Consistent with this, cellular ATP levels in ATPIF1 knockdown cells were ~50% lower than in control or PINK1 knockdown cells (Fig 2C). To confirm if ATPIF1 promotes PARK2 recruitment by direct effects on the ATP synthase, we monitored PARK2 recruitment in oligomycin-treated cells. Whereas addition of oligomycin restored PARK2 recruitment in the absence of ATPIF1, oligomycin had no effect in PINK1 knockdown cells (Fig 2D-E). Recent data indicate that loss of $\Delta\Psi$ blocks PINK1 mitochondrial import and its accumulation and retention in the outer mitochondrial membrane;[15, 31] thus our observations suggested that PINK1 most likely functions downstream of ATPIF1 and the ATP synthase. To confirm this, we performed western blot analysis of PINK1 levels in cells following CCCP treatment, and observed a complete inhibition of full-length PINK1 accumulation in ATPIF1 knockdown cells, which can be rescued by oligomycin (Fig 2F). Taken together, these data indicate there is an absolute requirement for the F_1 -ATPase action for restoration of $\Delta\Psi$ and inhibition of PARK2 recruitment in cells lacking ATPIF1, and furthermore that these events occur upstream of PINK1 activation. Consequently, in the absence of ATPIF1, the reverse reaction of ATP synthase is sufficient to overcome an acute effect of uncoupling by restoring $\Delta\Psi$, thus removing the signal for PARK2 recruitment. This role of ATPIF1 is essential, and explains how even in cells lacking mitochondrial DNA, mitochondria can maintain electrochemical potential.[32] Taken together, these data provide additional evidence that ATPIF1 is a critical rheostat for establishing the amplitude of $\Delta\Psi$.

The restoration of $\Delta\Psi$ via the F_1 -ATPase is sufficient to block PARK2 recruitment following exposure to CCCP. However, with higher doses of CCCP (20 μ M), loss of ATPIF1 is unable to prevent PARK2 recruitment, indicating that the capacity of ATPase to

generate H^+ and restore $\Delta\Psi$ is saturable (Fig S4). Therefore, to confirm our findings, we used antimycin A, an inhibitor of mitochondrial respiration that targets complex III of the electron transport chain, and observed that antimycin A also induced PARK2 recruitment (Fig S5A-B). Indeed, ATPIF1 knockdown served to block the loss of $\Delta\Psi$ as measured by TMRE (Fig 3A). This was consistent with a reduction in PARK2 translocation in ATPIF1 knockdown cells relative to control cells (Fig 3B-C). Furthermore, cotreatment with oligomycin restored recruitment and loss of $\Delta\Psi$ in ATPIF1 knockdown cells, confirming that the ATP synthase is the relevant target of ATPIF1 and a critical regulator of PARK2 recruitment in this setting. Identical results were observed in U2OS cells (Fig S5C).

Given the centrality of PINK1-PARK2 signaling to mitophagy, we sought to investigate the role of ATPIF1 in this process. To this end, we developed a quantitative cell-based FACS assay using cell lines that stably express yellow fluorescent protein (YFP) targeted to mitochondria via fusion of the mitochondrial targeting sequence from ornithine transcarbamoylase (OTC), a matrix protein, as a proxy for mitochondrial mass (Fig S6A).[33] In the absence of overexpressed PARK2, significant mitophagy did not occur in U2OS cells in response to CCCP (Fig S6B-C). OTC-YFP fluorescence was >100 times more intense than that of GFP-PARK2 as measured by FACS analysis (Fig S7), thus the contribution made by GFP-PARK2 to the OTC-YFP FACS signal did not interfere with mitophagy measurements. As U2OS cells displayed the most robust mitophagy phenotype, U2OS cells expressing GFP-PARK2 and the OTC-YFP reporter were treated with CCCP for 24 h to induce mitophagy (Fig 4A). We observed a 10-fold reduction in mitochondrial mass (as measured by loss of OTC-YFP signal) following CCCP treatment, which was blocked in PINK1 knockdown cells (Fig 4B). Furthermore, western blot analysis of these same cells

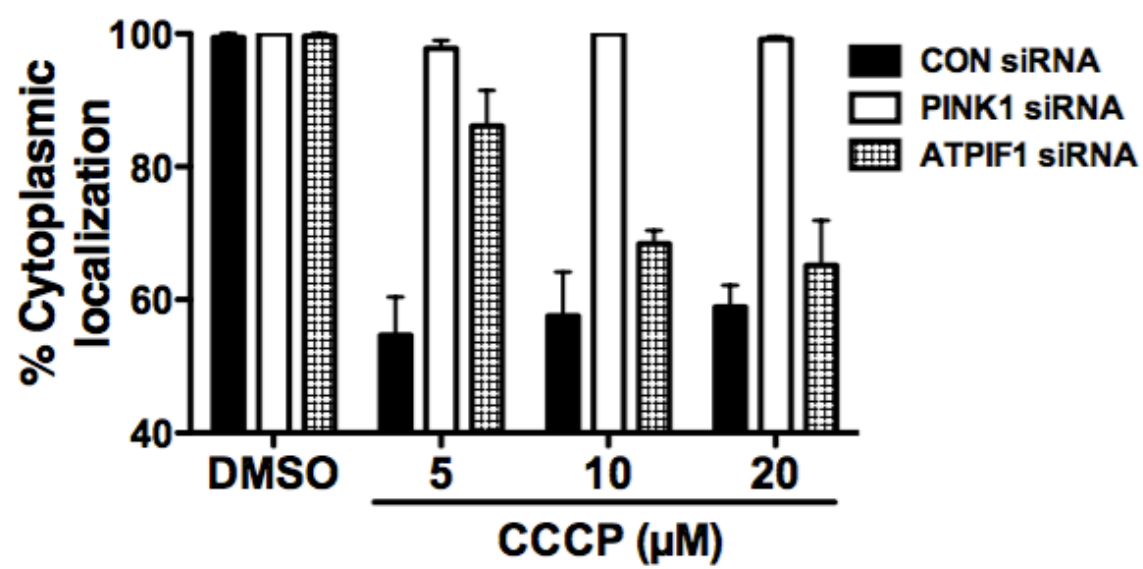


Fig S4: Histogram showing quantification of GFP-PARK2 recruitment to mitochondria in PINK1 and ATPIF1 knockdown cells. Effect of increasing dose of CCCP on recruitment of PARK2 is shown.

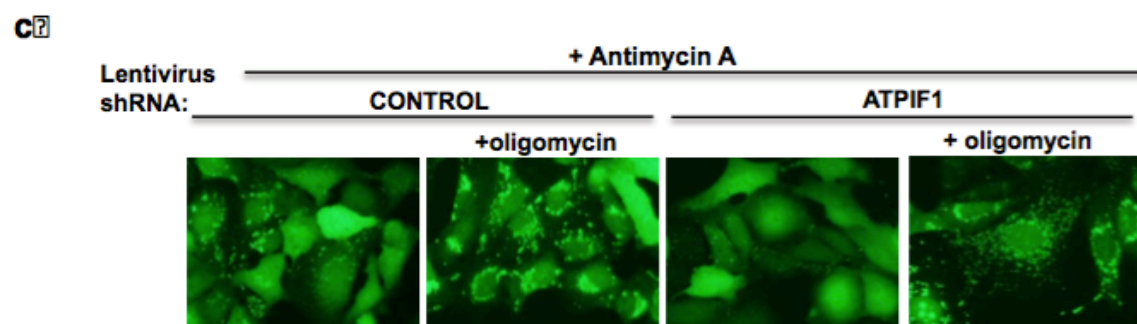
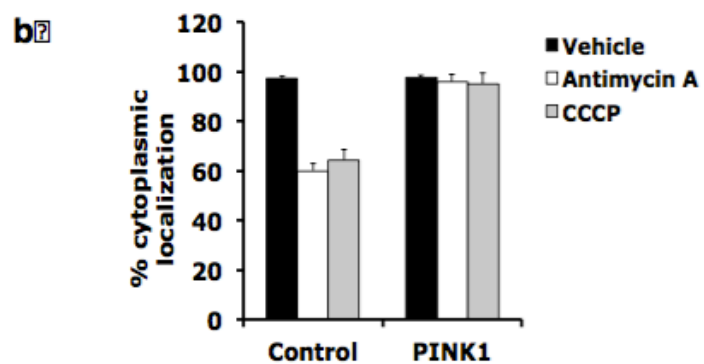
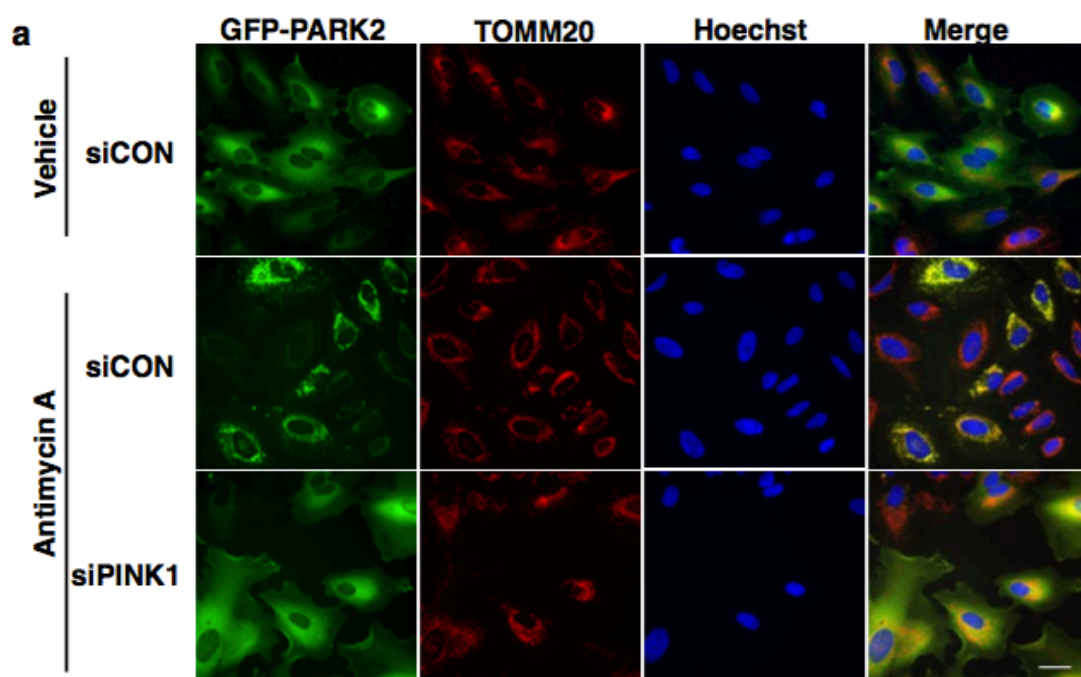


Fig S5: Antimycin A treatment induces GFP-PARK2 relocalization to mitochondria in control siRNA-treated cells, but not in PINK1 knockdown HeLa or U2OS cells. (A)

Fluorescence micrographs GFP-PARK2 localization in control and PINK1 knockdown cells following antimycin A (50 μ M) treatment. Nuclei are shown using Hoechst (blue).

Mitochondria are labelled with anti-TOMM20 antibody (red). Merge is shown at right. Scale bar, 25 μ m. (B) Histogram showing results from algorithm for automated detection of

regulators of GFP-PARK2 recruitment to mitochondria with PINK1 positive control. CCCP and antimycin A treatment induces GFP-PARK2 relocalization to mitochondria in control siRNA-treated cells, but not in PINK1 knockdown cells. (C) Fluorescence micrographs of GFP-PARK2 recruitment following antimycin A treatment (50 μ M) in U2OS cells infected with lentivirus expressing a non-targeting (CONTROL) or ATP1F1-directed shRNA. Effect of cotreatment with oligomycin is shown.

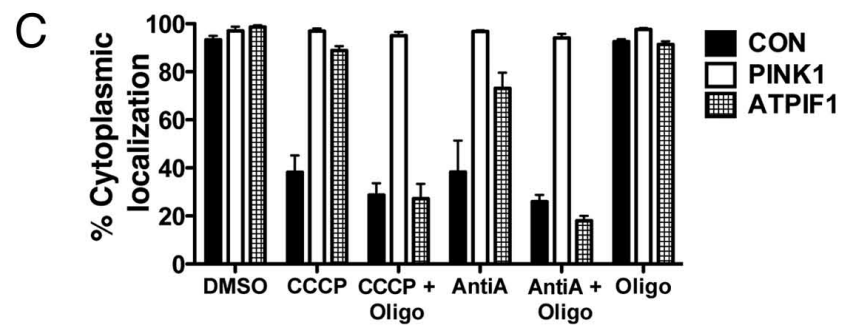
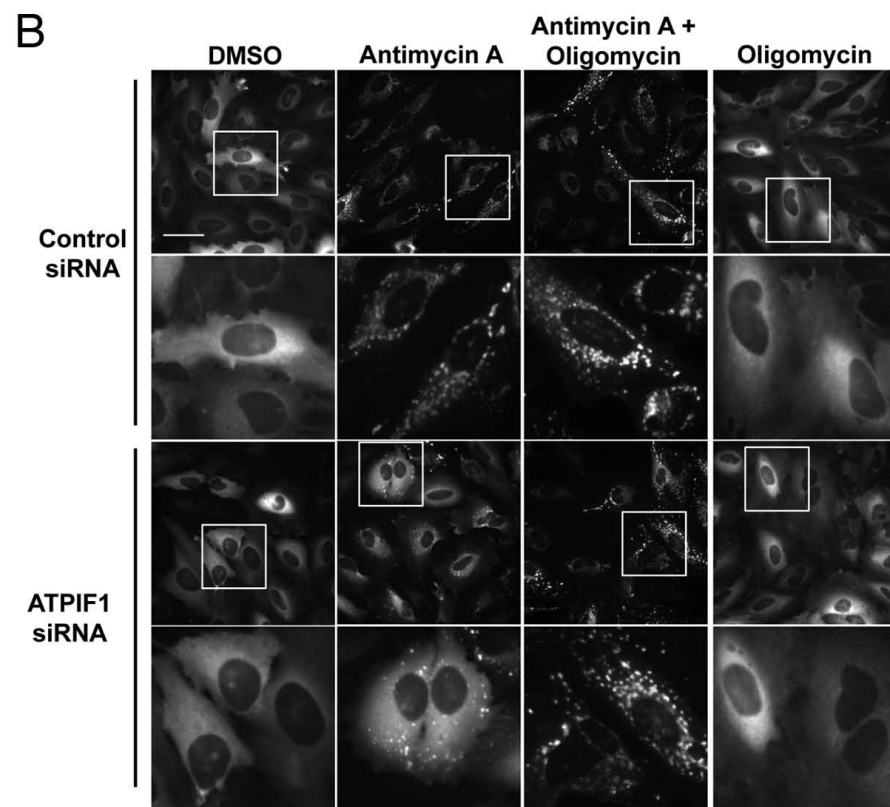
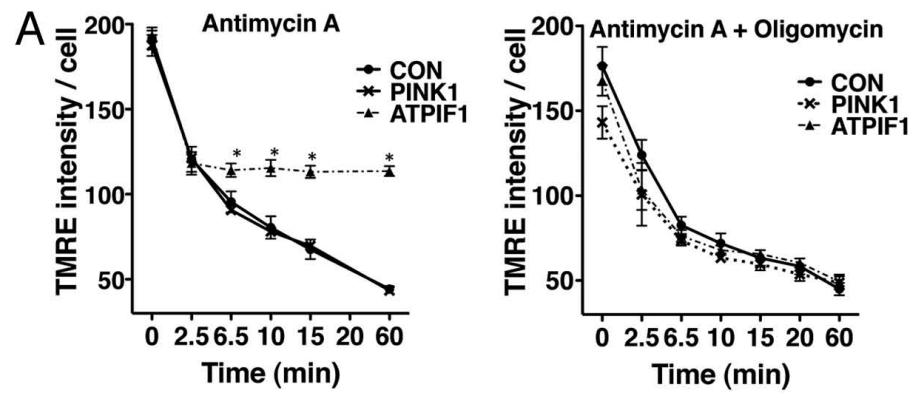


Fig 3: ATPIF1 is required for PARK2 recruitment triggered by inhibition of electron transport. (A) TMRE fluorescence intensity following treatment with complex III inhibitor antimycin A (left) or antimycin A + oligomycin (right) in control, ATPIF1 or PINK1 knockdown cells. Data are mean $\pm$ SD of triplicate measurements of 1 experiment that is representative of 3 independent experiments. * $P < 0.001$, paired t-test. (B) Fluorescence micrographs of GFP-PARK2 in control and ATPIF1 knockdown HeLa cells treated for 1 h with 40 μ M antimycin A and 2.5 μ M oligomycin alone or together. Scale bar: 50 μ m. Bottom row for control and ATPIF1 siRNAs is the inset magnified 4 $\times$. (C) Histogram showing quantitation of GFP-PARK2 localization in control, ATPIF1 or PINK1 knockdown cells following treatment with CCC P, antimycin A and oligomycin, alone or in the indicated combinations. Data are mean $\pm$ SD of 3 independent experiments.

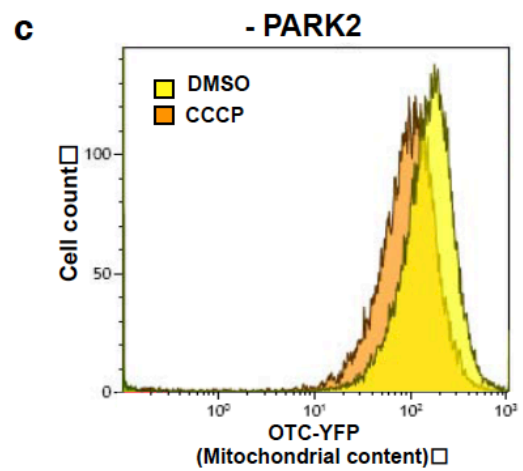
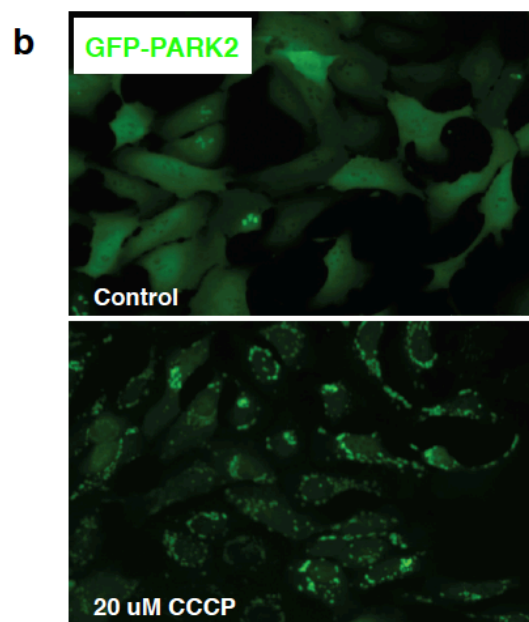
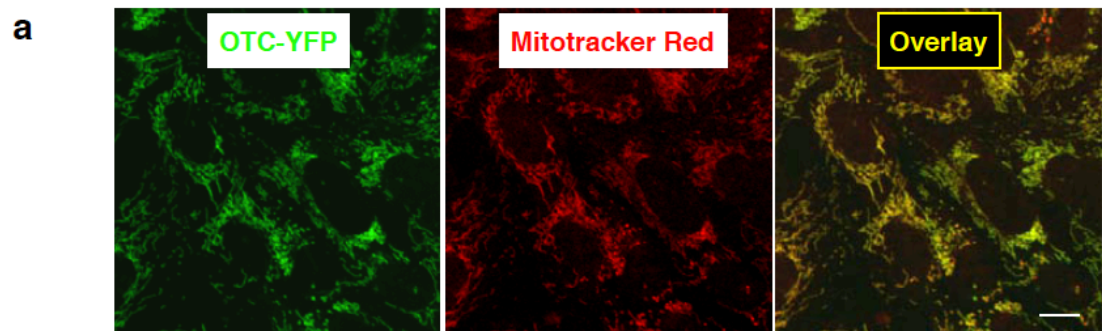


Fig S6: Development of a FACS-based assay for mitophagy. (A) Confocal fluorescence micrographs showing mitochondrial localization of YFP in the OTC-YFP stable cell line. MitoTracker Red staining confirms the mitochondrial localization of OTC-YFP. Overlay is shown at right. Scale bar, 25 μm . (B) GFP-PARK2 recruitment in response to 1 h treatment with 10 μM CCCP in U2OS cells. Scale bar, 50 μm . (C) FACS analysis showing minimal mitochondrial loss following 24 h 10 μM CCCP treatment in control U2OS cells in the absence of overexpression of GFP-PARK2.

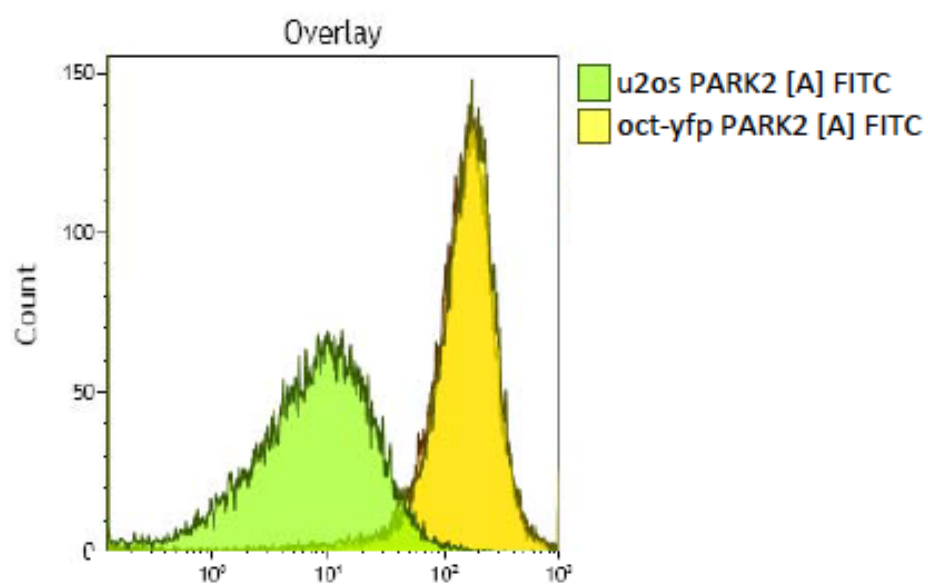


Fig S7: FACS profiles showing relative fluorescence signal intensity of U2OS cells expressing GFP-PARK2 or U2OS cells OTC-YFP. OTC-YFP signal is >100-fold higher than that of GFP-PARK2.

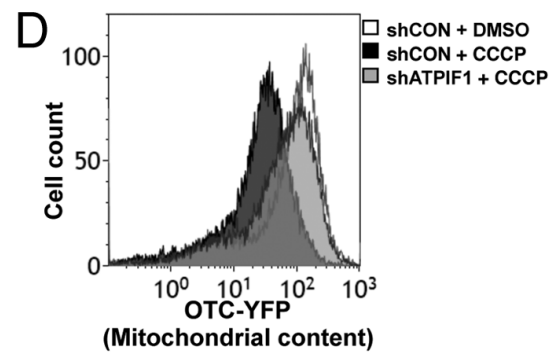
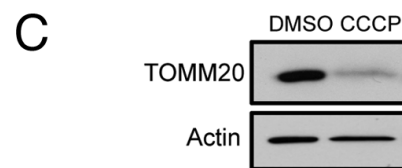
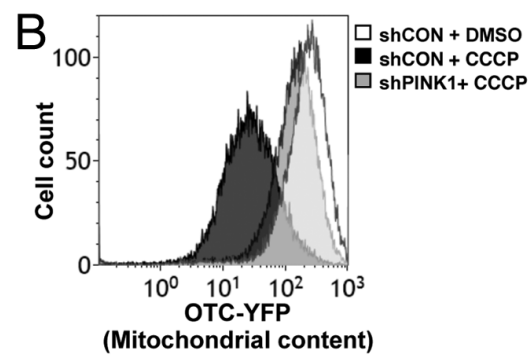
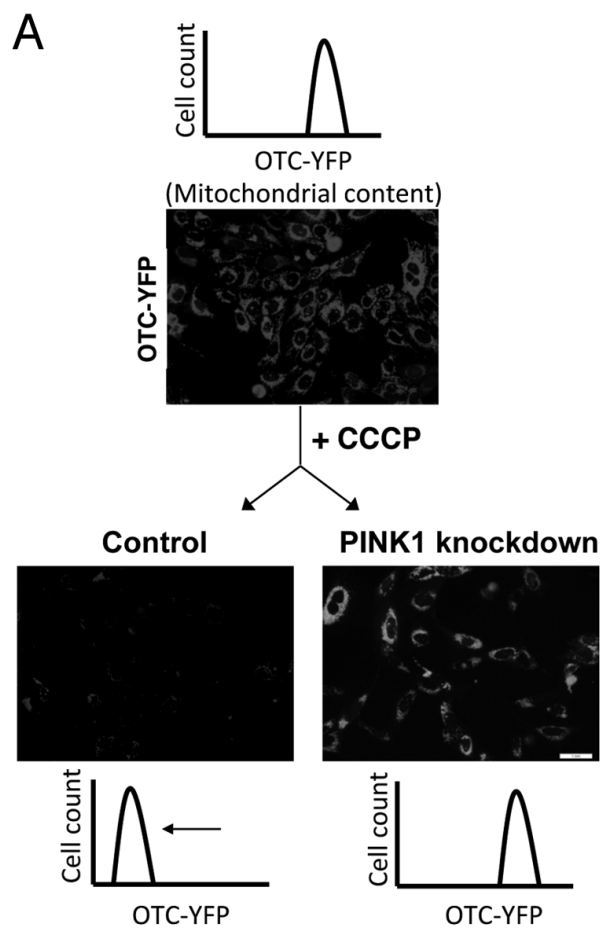


Fig 4: ATPIF1 is required for mitophagy. (A) Outline of FACS-based mitophagy assay.

U2OS cells stably expressing OTC-YFP were generated to monitor changes in mitochondrial content by FACS analysis. Following 24 h treatment with 10 μ M CCCP, cells expressing GFP-PARK2 undergo mitophagy and lose OTC-YFP signal. Scale bar: 50 μ m. (B) FACS profiles showing effect of 24 h treatment with DMSO vehicle or 10 μ M CCCP on OTC-YFP levels as a proxy for mitophagy in control (shCON) or PINK1 knockdown cells (shPINK1). Y-axis is a log scale. (C) Western blot analysis of TOMM20 in cell extracts following 24 h treatment with 10 μ M CCCP. Actin loading control is shown. (D) FACS profiles showing effect of 24 h treatment with 10 μ M CCCP on mitophagy in control (shCON) or ATPIF1 knockdown (shATPIF1) cells.

revealed a loss of TOMM20 protein, validating our FACS-based approach (Fig 4C). Thus, mitophagy triggered by activation of the PINK1-PARK2 pathway is faithfully reproduced in this system. Mitophagy was also impaired in IF1 knockdown cells, indicating that both PARK2 recruitment and mitophagy in U2OS cells required ATPIF1 (Fig 4D).

Human patients with mitochondrial myopathies progressively accumulate mutated mtDNA, suggesting that quality control mechanisms fail to compensate for dysfunction in the long term.[34] To examine the effects of ATPIF1 loss in a setting of mitochondrial dysfunction, we examined whether or not ATPIF1 plays a role in mitochondrial quality control in Rho0 cells, which have impaired mitochondrial respiration due to a lack of electron transport capacity. TMRE-stained Rho0 cells displayed a lower resting $\Delta\Psi$ than control 143B cells, that was dramatically decreased following expression of wild-type but not mutant ATPIF1 using lentivirus (Fig 5A, Fig S8A). MitoTracker Green staining was not affected, indicating that the loss of potential in the Rho0 cells was not the result of a loss of mitochondrial mass (Fig S9). Notably, dissipation of $\Delta\Psi$ in 143B cells required a higher dose of CCCP than for Rho0 cells, likely due to the contribution to $\Delta\Psi$ from intact electron transport. Loss of $\Delta\Psi$ in Rho0 cells following restoration of ATPIF1 indicates that Rho0 cells rely solely on ATPase activity to maintain $\Delta\Psi$ under normal conditions. Although Rho0 cells lack subunits MT-ATP6/A and MT-ATP8/A6L of the ATP synthase encoded by mtDNA, they still are capable of forming a functional F_1 -ATPase that serves to generate membrane potential and also binds ATPIF1.[32, 35, 36] [ENREF_1](#) Consistent with this, Rho0 cells revealed a 4-fold reduction in ATPIF1 levels by western blot compared to control 143B cells (Fig 5B-C). In this cellular context, GFP-PARK2 recruitment appeared as focal spots of GFP-PARK2 that colocalized with TOMM20 instead of global recruitment.

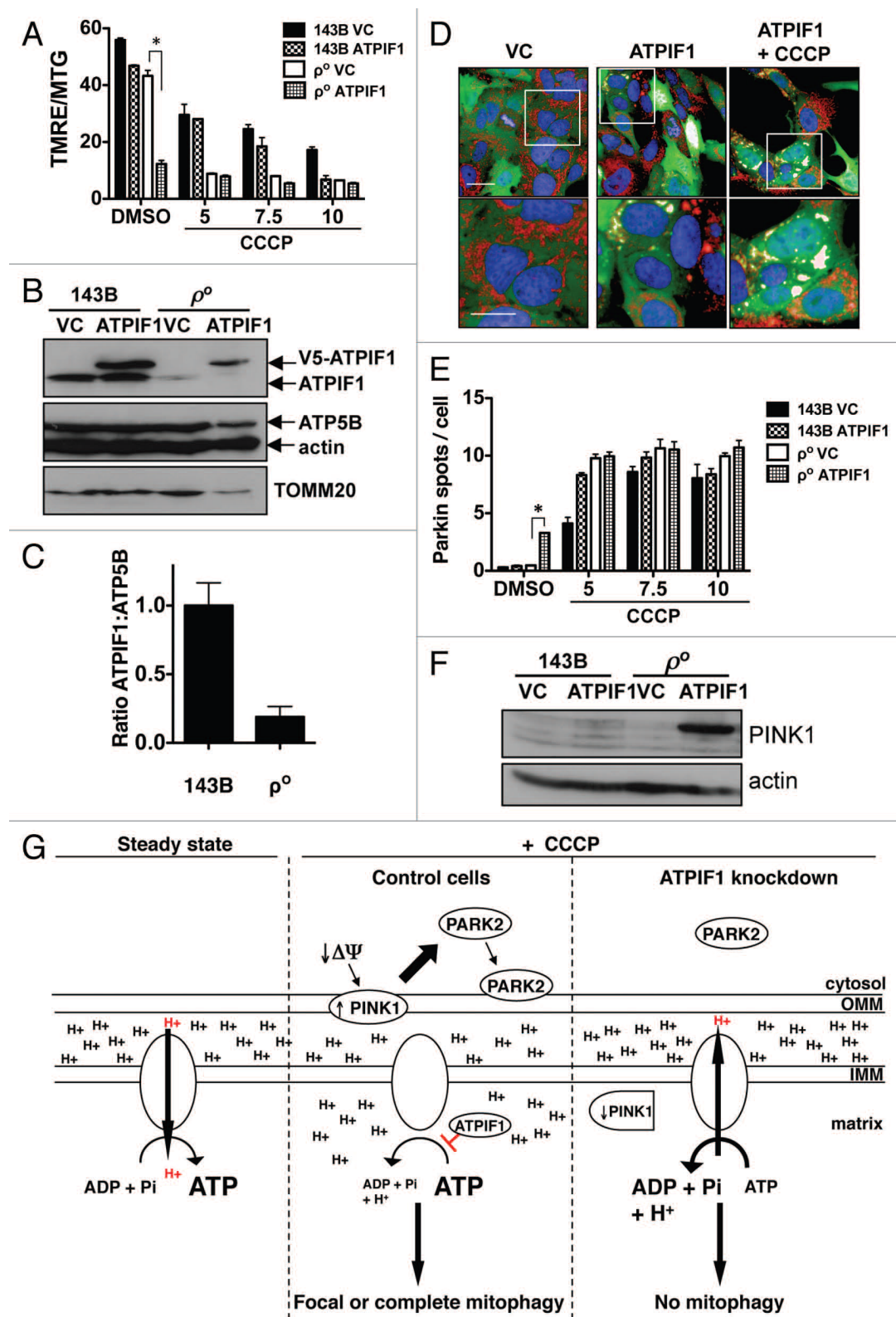


Fig 5: Restoration of PARK2 recruitment in Rho0 cells expressing ATPIF1. (A) TMRE staining of 143B control and Rho0 cells infected with control or ATPIF1-expressing lentivirus. Effect of treatment with DMSO vehicle and 5, 7.5, or 10 μ M CCCP is shown. Data are mean $\pm$ SD of 3 independent experiments. *P < 0.001, paired t-test. (B) Western blot showing levels of ATPIF1 in 143B control and Rho0 cells. (C) Histogram showing ratio of ATPIF1 protein:F1Fo-ATP synthase ATP5B subunit measured by densitometry. Data are mean $\pm$ SD of 4 individual samples. (D) Fluorescence micrographs of GFP-PARK2 in cells infected with control (VC) or lenti-ATPIF1. Effect of 5 μ M CCCP treatment is shown. Bottom row is a 2.3 $\times$ magnification of inset in top. Scale bar: 50 μ m (top) and 125 μ m (bottom). (E) Histogram showing GFP-PARK2 spots on mitochondria in 143B and Rho0 cells infected with control (VC) or lenti-ATPIF1 as determined by imaging analysis using Columbus software. Data are mean $\pm$ SD of 3 independent experiments. *P < 0.05, paired t-test. (F) Western blots showing level of PINK1 in control (VC) or lenti-ATPIF1 infected 143B and Rho0 cells. Actin is shown as loading control. (G) Model to summarize the role of ATPIF1 in PARK2 recruitment. At steady-state, potential across the IMM ($\Delta\Psi$) is high and the F1Fo-ATP synthase generates ATP. Following CCCP treatment, potential is lost due to equilibration of protons (H^+), activating the reverse (ATPase) reaction of the synthase. In control cells, ATPIF1 blocks the ATPase activity, promoting loss of $\Delta\Psi$ and activation of the PINK1-PARK2 mitophagy pathway. High cellular ATP levels are maintained (indicated by larger font). In the absence of ATPIF1, the ATPase generates protons to restore $\Delta\Psi$ at the expense of cellular ATP (smaller font). As a consequence, PINK1 accumulation, PARK2 recruitment and mitophagy are all blocked.

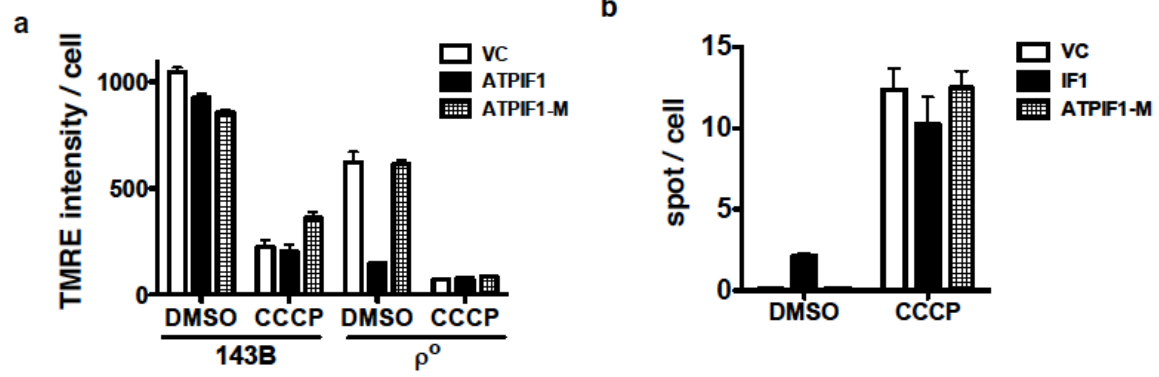


Fig S8: ATP1F1 interaction with F1-ATPase is required for PARK2 recruitment and loss of potential in Rho0 cells. Cells were infected with a lentivirus expressing siRNA-resistant ATP1F1 cDNA mutated at E55 and Y58 (IF1-M) for 72 h. The wild-type siRNA-resistant ATP1F1 cDNA (IF1) and a vector control (VC) lentivirus were used as control. (A) TMRE intensity of 143B cells (control) and Rho0 cells was measured 72 h after lentiviral infection and a 1 h treatment with 10 μ M CCCP or DMSO control. (B) The number of PARK2 foci in Rho0 cells expressing GFP-PARK2 and IF1 or IF1-M was assessed by automated microscopy following a 1 h treatment with 10 μ M CCCP. All data are presented as triplicates $\pm$ SD and are representative of two independent experiments.

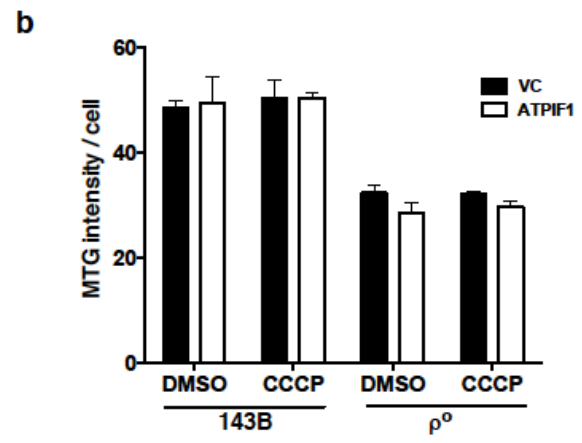
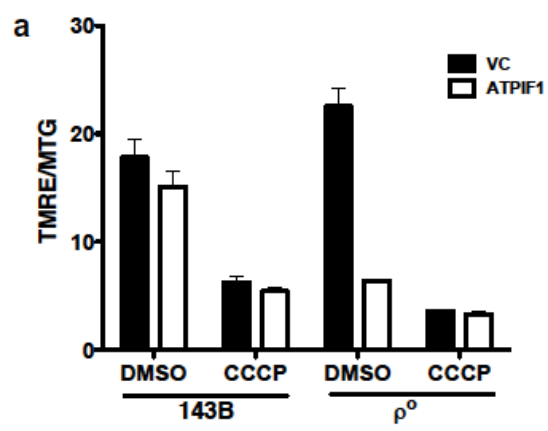


Fig S9: Restoration of ATP1F1 in Rho0 cells reduces mitochondrial potential without affecting mitochondrial mass. (A) Histogram showing the ratio of TMRE intensity per cell in parental 143B and 143B-Rho0 cells following restoration of ATP1F1. TMRE intensity is plotted relative to MitoTracker Green. (B) Histogram showing MitoTracker Green intensity/cell for cells shown in (A). For (A) and (B), cells were preloaded with TMRE, MitoTracker Green, and Hoechst and then treated for 1 h with 5 μ M CCCP.

Importantly, restoration of wild-type but not mutant ATPIF1 protein in Rho0 cells triggered PARK2 recruitment (Fig 5D and E, Fig S8B) as well as selective loss of TOMM20 and the ATP5B/b subunit of the ATP synthase. The increase in PARK2 recruitment in Rho0 cells expressing ATPIF1 was consistent with an accumulation of full-length, or active, PINK1 (Fig 5F). Taken together, these data indicate that re-expression of ATPIF1 in Rho0 cells is sufficient to induce the mitophagic program.

Discussion

We employed an unbiased strategy to identify genes involved in PINK1-PARK2-mediated mitophagy. Using RNAi technology to screen over 18,000 genes in the human genome, we identified a unique requirement for ATPIF1 in order to initiate mitophagy. An unresolved question that has emerged from studies of the role of PINK1 and PARK2 in mitophagy is how often do mitochondria lose potential under steady-state conditions? Mitochondria experiencing fluctuations in $\Delta\Psi$ appear to employ respiration and reversal of ATP synthase to counteract loss of $\Delta\Psi$, and thus avoid clearance by mitophagy. ATPIF1 regulates the loss of potential across the IMM through its inhibition of the reversal of the ATP synthase. In cases of mitochondrial stress, if the ATPase is blocked either by ATPIF1 or with oligomycin treatment, potential is lost and full-length PINK1 can be stabilized on the outer mitochondrial membrane. This is a critical step in recruiting PARK2 to damaged mitochondria and activating mitophagy. Taken together, our studies reveal the essential role played by ATPIF1 in regulating the activity of the ATP synthase, highlighting this inhibitor as a rheostat that defines the electrochemical potential and a critical upstream regulator of the PINK1-PARK2 pathway (Fig 5G).

Rho0 cells lack mitochondrial DNA, preventing them from forming a functional ETC. Given the importance of maintaining potential in mitochondria to maintain other functions such as protein import, these cells must rely on the reversal of the ATP synthase. Loss of ATPIF1 in Rho0 cells is perhaps indicative of a cellular requirement for carrying energetically deficient mitochondria over none at all. Importantly, this may also explain why dysfunctional mitochondria accumulate in patients with mitochondrial disease. We show that restoration of ATPIF1 in Rho0 cells does indeed activate PARK2 recruitment, providing evidence that ATPIF1 could be used to promote the clearance of defective mitochondria. Future studies will look at the effect of ATPIF1 overexpression in patient cell lines with heteroplasmic levels of mitochondrial mutations to further investigate this possibility.

Is PARK2-mediated mitophagy a rare occurrence *in vivo*? Mitochondria transiently lose potential during many critical functions, such as during muscle contraction when calcium waves propagate between mitochondria and the endoplasmic reticulum. Recently, pacing of the dopaminergic neurons has been shown to involve transient activation of the uncoupling proteins, which act like CCCP to depolarize the inner membrane.[37] The regulation of both forward and reverse activities of the ATP synthase in the pacing of the organelles in a variety of cell models is understudied. Interestingly, a recent study demonstrated that Bcl-xL suppresses large fluctuations in mitochondrial potential via direct interactions with the β subunit of ATP synthase, as does ATPIF1,[38] raising the possibility that suppression of apoptosis by BCL2L1 may in part involve stabilization of $\Delta\Psi$. [39] Taken together, our work places ATPIF1 and mitochondrial ATP synthase as critical upstream elements in the PINK1-PARK2-mediated mitophagy pathway and provides genetic evidence that loss of $\Delta\Psi$ is a critical trigger for PARK2 recruitment to mitochondria. Given the

possibility that failure of mitophagy may underlie other human diseases, our work has the potential to inform PD and other disorders.

Materials and Methods

Reagents.

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP, C2759), oligomycin (75351), N-acetyl cysteine (NAC, A7250), and staurosporine (S6942) were from Sigma. MitoTracker Red CM-H₂XRos (M7513), MitoTracker Green FM (M7514), TMRE (T669), and Hoechst 33342 (H3750) were from Invitrogen/Molecular Probes. Antibodies were from the following sources: TOMM20 (Santa Cruz, sc11415), ATP1F1 (Sigma, SAB2100188), beta actin (Sigma, A5441), PINK1 (Novus, BC100-494), LRRK2 (MJFF2, Epitomics, 3514-1), ATP5B (3D5) (Abcam, ab14730).

Cell culture.

HeLa cells (P34-36) and 143B cells were cultured in DMEM with 25 mM glucose at 37°C in a humidified incubator with 5% CO₂. Rho0 (derived from 143B) medium was supplemented with 1 mM pyruvate and 50 µg/ml uridine. U2OS cells were cultured in McCoy's 5a medium (Gibco, 16600-082). All media contained 10% FBS and streptomycin/penicillin.

Imaging screen.

Pools of four siRNA SMARTpool duplexes targeting 692 genes (Qiagen) or 18,255 human genes (Dharmacon siGenome) were spotted into 384-well plates. 10 nM of siRNA was

reverse transfected into 450 HeLa cells using 0.025 μ l of Lipofectamine RNAiMAX (Invitrogen, 13778). Coincident with transfection, the cells were infected with an adeno-associated virus (AAV) expressing N- terminally tagged GFP-PARK2. 72 h later, 5 μ M CCCP or vehicle (DMSO) was added to the cells for 1 h prior to fixation. Cells were washed with PBS, permeabilized with 0.1% Triton X-100 for 15 min, and subsequently stained with rabbit anti-TOMM20 primary antibody and AlexaFluor goat anti-rabbit secondary antibody (Sigma-Aldrich; A11012). Images of 10 fields per well were collected using a Cellomics Arrayscan Vti with a 40x objective and an XF53 filter set. Nuclei were counterstained with 1 μ g/mL Hoechst 33342 and cell boundaries were approximated using a 40-pixel radius from the edge of the nucleus or the midpoint between two neighboring nuclei. Cells expressing GFP-PARK2 were analyzed using Thermo Scientific's Cellomics vHCS:Scan software to differentiate cytoplasmic and mitochondrial GFP-PARK2 signal. Each well was then scored based on the percentage of cells containing cytoplasmic GFP-PARK2. The level of GFP-PARK2 associated with the mitochondria, as designated by TOMM20 fluorescence, was measured using the Matthews correlation coefficient of the two signals. A cutoff value for the correlation coefficient was set where greater than 85% of DMSO-treated cells were considered as having only cytoplasmic GFP-PARK2. Following algorithm analysis, images from hit wells were manually inspected to verify failure of PARK2 mitochondrial recruitment.

Mitophagy assay.

Stable clones of HeLa and U2OS cells expressing YFP tagged at the N terminus with the mitochondrial targeting sequence from OTC were pooled and FACS-sorted to generate

uniform OTC-YFP populations. To measure mitophagy, cells were infected with AAV-GFP-PARK2 on day 0 and treated on day 3 with 10 μ M CCCP. On day 4, cells were trypsinized, pooled, and analyzed for OTC-YFP signal by FACS. U2OS cells were chosen for all analyses owing to their superior infection rate with lentivirus-encoding shRNA. To label mitochondria in live cells, MitoTracker Red CM-H2Xros (300 nM) was added to culture medium for 30 min at 37°C, prior to imaging with an Olympus confocal microscope equipped with LG-ESF200 CCD camera and analyzed using Olympus Fluoview software.

ATP assay.

siRNA knockdown was conducted in a white opaque 384-well plate as detailed above for the imaging screen. 72 h later, wells were treated for 1 h with 5 μ M CCCP or DMSO. Staurosporine treatment (1 μ M for 3 h) was included as a positive control for ATP depletion. The plate was equilibrated at room temperature for 30 min prior to the addition of an equal volume of Cell-Titre Glo (Promega, G7570). Contents were mixed for 2 min at 600 rpm on a microplate shaker and equilibrated for 10 min. Luminescence values were determined using a Synergy2 plate reader (BioTek) and normalized based on cell number using Hoechst nuclear stain.

$\Delta\Psi$ measurements.

MitoTracker Red staining was achieved by incubating cells with 300 nM dye for 30 min following treatment with DMSO or CCCP. Cells were then fixed and stained for imaging as in the imaging screen. TMRE measurements were performed by live imaging on the Perkin

Elmer Opera automated confocal microscope acclimatized to 37°C and 5% CO₂ and using a 20X water objective. Cells were incubated with 40 nM TMRE and 1 µg/ml Hoechst for 30 min prior to imaging and treatment. TMRE intensity per cell was evaluated with Columbus software (Perkin-Elmer) using an algorithm to look at the signal intensity of TMRE within a 30-pixel radius from the nucleus.

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Appendix 2

Genome-wide RNAi Screen Identifies ROMO1 as an Essential Redox-Dependent Regulator of Mitochondrial Dynamics

Preface

Appendix 2 contains a published paper on *ROMO1*, which we have uncovered in a genome screen for genes required for normal mitochondrial morphology. Silencing of *ROMO1* led to mitochondrial fragmentation due to a loss of OPA1 oligomers. Oxidative stressor menadione inactivates Flag-ROMO1 protein by inducing the formation of ROMO1 high molecular weight disulphide-linkages. ROMO1 is further shown to be required for spare respiratory capacity, but the mechanism of this is unclear. Taken together, these data show that ROMO1 is a novel regulator of mitochondrial dynamics.

Author list

Matthew Norton, Andy Cheuk-Him Ng, Stephen Baird, Ariane Dumoulin, Timothy Shutt, Nancy Mah, Miguel A. Andrade-Navarro, Heidi M. McBride, Robert A. Screaton.

Author contribution

MN performed all of the experiments in this manuscript. AC-HN, with the assistance of AD, performed the genome screen that identified *ROMO1*. SB helped with the quantitation of fluorescence images. MN supervised the study and wrote the manuscript.

Abstract

The dynamics of mitochondria undergoing fusion and fragmentation govern many mitochondrial functions, including regulation of cell survival. Although the machinery that catalyzes fusion and fragmentation has been well described, less is known about the signaling components that regulate these phenomena. We performed a genome-wide RNA interference (RNAi) screen and identified reactive oxygen species modulator 1 (ROMO1) as a redox-regulated protein required for mitochondrial fusion and normal cristae morphology. We show that oxidative stress promoted the formation of high-molecular weight ROMO1 complexes and that knockdown of ROMO1 promotes mitochondrial fission. ROMO1 was essential for the oligomerization of the inner membrane guanosine triphosphatase (GTPase) OPA1, which is required to maintain the integrity of cristae junctions. As a consequence, cells lacking ROMO1 displayed fragmented mitochondria and loss of cristae, causing impaired mitochondrial respiration and increased sensitivity to cell death stimuli. Together, our data identify ROMO1 as a critical molecular switch that couples metabolic stress and mitochondrial morphology, linking mitochondrial fusion to cell survival.

Introduction

Mitochondria form a dynamic reticulum that is sculpted by cycles of ongoing fusion and fission events of individual organelles, a phenomenon known as mitochondrial dynamics [1-3]. Several mitochondrial shaping proteins that catalyze the opposing processes of membrane fusion and fission have been identified. These include MFN1 and MFN2 as well as OPA1 in mammals (Fzo1 and Mgm1 in yeast) [4] which are required during fusion for the integration of outer and inner mitochondrial membranes, respectively, and DRP1 (Dnm1), which catalyzes fragmentation following recruitment to mitochondria by MFF [5, 6]. DRP1 is required for cell death during development [7], as well as for focal delivery of energy at neuromuscular junctions [8], and is related to neurodegeneration and ischemia/reperfusion in the heart [9-12]. Mutations in MFN2 underlie Charcot-Marie-Tooth 2a [13] while OPA1 mutations are associated with autosomal dominant optic atrophy [14]; downregulation of MFN2 also occurs in obesity-related insulin-resistance associated with type 2 diabetes [15], highlighting the pathological consequences of dysfunctional mitochondrial dynamics [16]. During programmed cell death the mitochondrial network fragments to facilitate opening of junctions of inner mitochondrial membrane cristae and the consequent metabolic catastrophe [3, 17-21]. In contrast, mitochondrial fusion following starvation or stress is thought to attenuate mitophagy and/or apoptosis [22-25]. Regulated interactions between MFN2 and BAX and BAK, two pro-apoptotic members of the Bcl2 family, appear to govern the earliest events of the apoptotic cascade, demonstrating an intimate relationship between mitochondrial morphology, integrity and cell fate [26-31]. However, which signals and protein effectors control the activities of the shaping proteins, and how mitochondrial dynamics is integrated into cell death signaling cascades is still unclear [32, 33].

Results

Genome-wide RNAi screen identifies ROMO1 as a novel regulator of mitochondrial fusion

To identify regulators of mitochondrial morphology, we performed a genome-wide RNAi imaging screen in HeLa cells (Fig S1A), using positive control siRNAs targeting DRP1 to induce elongation or a pool of siRNA targeting MFN1 and MFN2 to induce fragmentation (Fig 1A). We applied an algorithm that calculates the length:width or the aspect ratio of mitochondria in screen images and reproducibly distinguished between control, fused, and fragmented mitochondria (Fig 1B). Many regulators of mitochondrial dynamics, including DRP1, OPA1, MFF and YMEL1, were identified by the screen (Fig 1C). siRNA pools that generated robust Z-scores within the range observed for the internal siRNA controls were considered candidates for validation. Three out of four siRNAs targeting reactive oxygen species modulator 1 (ROMO1) produced robust fragmentation (Fig 1D-E), identifying it as a top candidate. ROMO1 is a 79 amino acid mitochondrial transmembrane protein that was originally identified as a positive regulator of mitochondrial ROS production [34-37], proliferation [38, 39], and senescence [35].

To rule out off-target effects of ROMO1 siRNA, we introduced an siRNA-resistant ROMO1 cDNA into U2OS cells using lentivirus which restored the normal network aspect ratio in ROMO1 knockdown cells (Fig 1F-G, Fig S1B) and validated knockdown at the mRNA level by qPCR (Fig S1C). We next generated a mutant ROMO1 cDNA (ROMO1-FFAA) carrying two Ala substitutions at two highly conserved Phe residues, Phe⁶⁷ and Phe⁷⁰. This mutant (FFAA) could not rescue normal morphology in cells lacking ROMO1. Next we

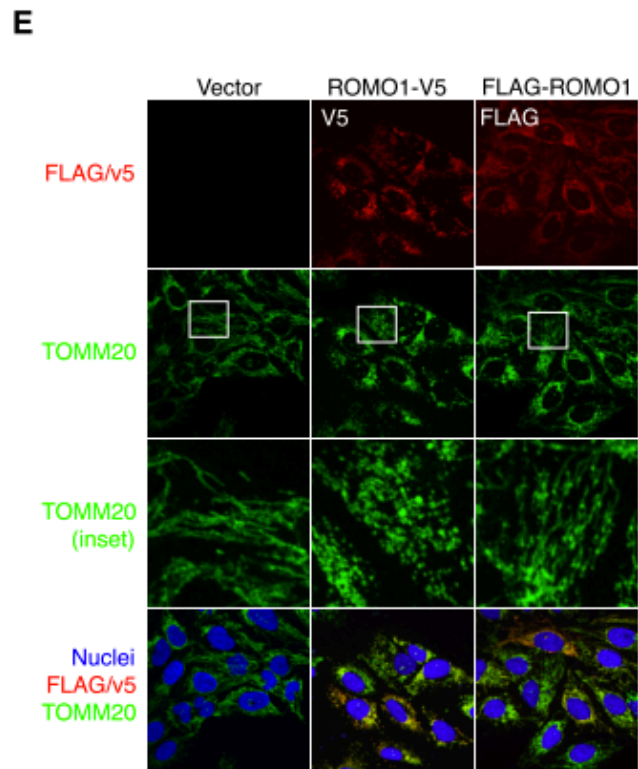
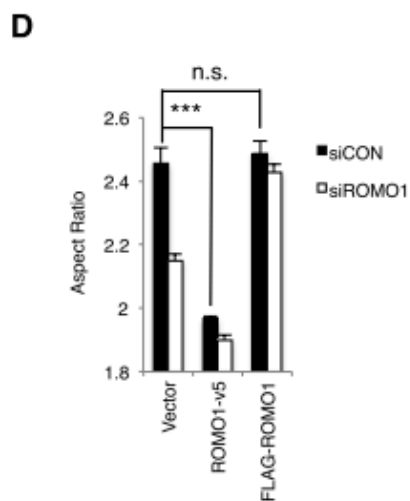
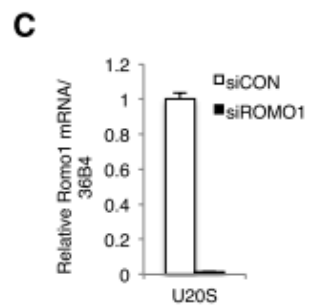
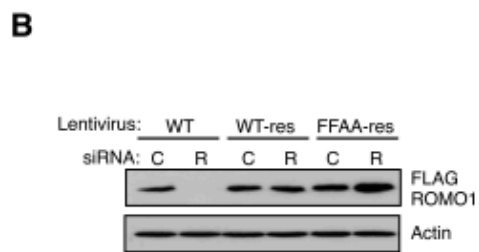
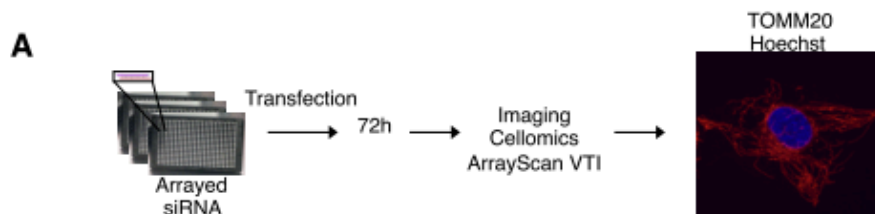


Fig S1: The high throughput mitochondrial morphology assay. HeLa cells cultured in 384 well plates were stained with TOMM20 antibody and Hoechst to monitor the status of the mitochondrial network and nuclei, respectively, and imaged on a Cellomics Vti automated microplate imager 3 days after reverse transfection with Dharmacon siRNA SmartPool library.

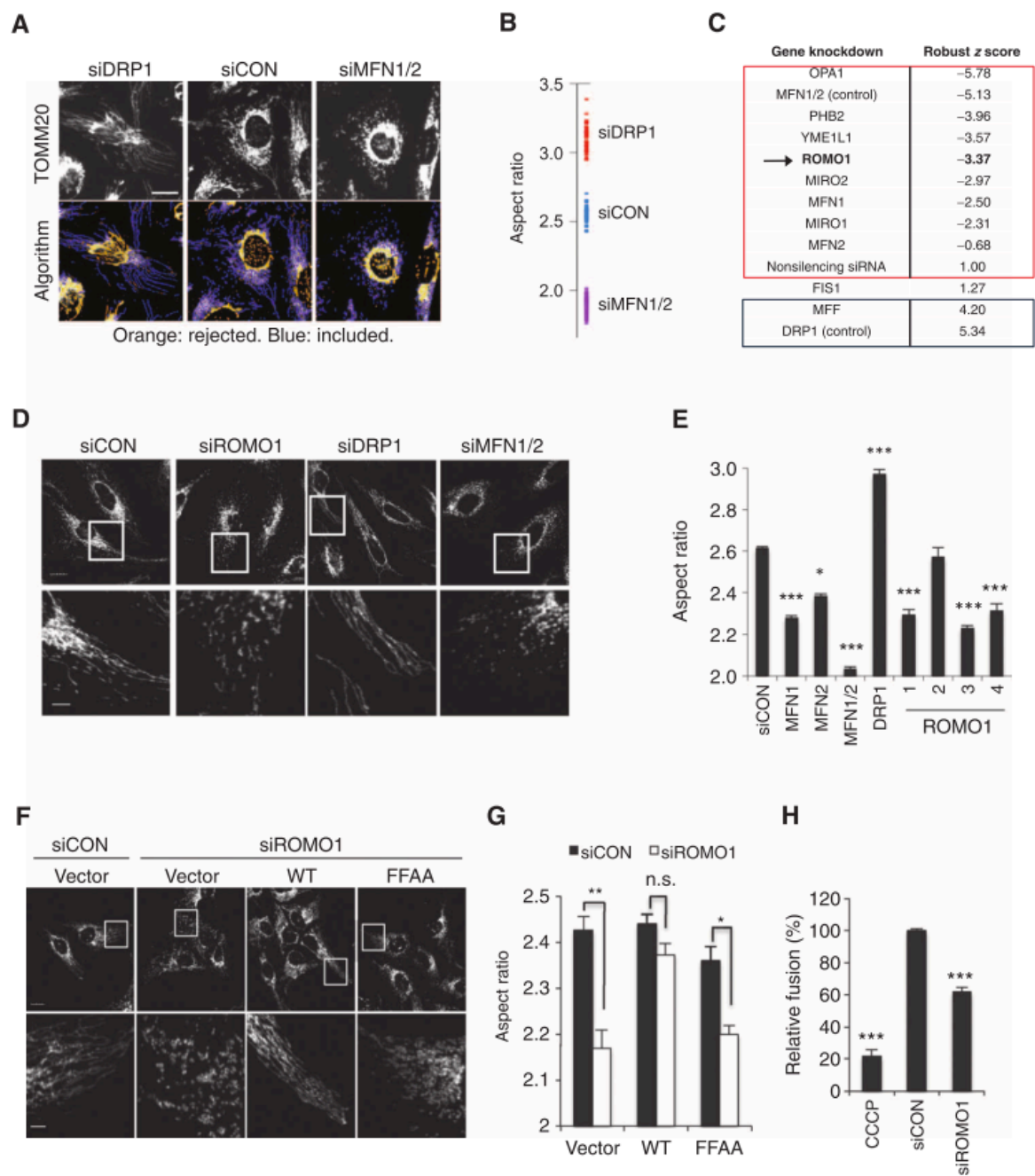


Fig 1: Genome-wide RNAi imaging screen identifies ROMO1 as a novel regulator of mitochondrial fusion. A. Representative fields of control, fused (siDRP1), or fragmented phenotypes (siMFN1/2) are shown (mag = 40X). Regions highlighted in orange were rejected by the algorithm to avoid perinuclear condensation artifacts. Images are representative of 3 independent experiments and 50 cells per condition. B. Scatter plot of Length:Width (aspect) ratio as determined by the algorithm shows robust separation of cells with control, fused (DRP1 siRNA), and fragmented (MFN1/2 siRNA) mitochondria. C. Table showing the Robust Z-score, the distance of the value from the median in standard deviation units, of ROMO1 and known regulators of mitochondrial dynamics in the RNAi screen. D. Photomicrographs of HeLa cells transfected with the indicated siRNAs. Scale bar = 20 nm. Inset shown at bottom, scale bar = 5 nm. Images are representative of 3 independent experiments and 50 cells per condition. E. Histogram showing quantitation of aspect ratio for the individual ROMO1 siRNAs used as a pool in D and for knockdown of MFN1 and MFN2 as well as DRP1 controls. Error bars represent s.e.m of 3 independent experiments. Statistical significance was determined by two-tailed unpaired *t*-test (* $p < 0.05$, *** $p < 0.001$) F. Rescue of ROMO1 knockdown phenotype with siRNA-resistant ROMO1 WT cDNA, but not with the non-functional ROMO1-FFAA mutant (in which Phe⁶⁷ and Phe⁷⁰ were mutated to Ala) and vector control. Images are representative of 3 independent experiments and 50 cells per condition. G. Histogram showing the aspect ratio for cells expressing empty vector or siRNA-resistant WT and FFAA constructs in control and ROMO1 knockdown cells shown in A. * $p < 0.05$, ** $p < 0.01$, n.s.: not significant. H. *In vitro* mitochondrial fusion assay showing luciferase values generated using mitochondria from cells transfected with control or ROMO1 siRNA or cells treated with CCCP. N=3 independent biological replicates, *** $p < 0.001$.

analyzed *in vitro* fusion rates of mitochondria isolated from control and ROMO1 knockdown cells using a luciferase complementation assay [40]. Mitochondria lacking ROMO1 showed a 50% reduction in fusion rate compared to controls (Fig 1H), indicating that loss of ROMO1 results in fragmentation of the mitochondrial network due to a reduction in fusion. While previous work has suggested that ROMO1 overexpression drives mitochondrial fragmentation [37], this is likely due to an artifact, as cells expressing a ROMO1 construct tagged at the C-terminus and not the N-terminus display fragmentation, suggestive of a dominant negative effect due to a tag at the C-terminus (Fig S1D-E). Taken together, our data demonstrate that ROMO1 is required for mitochondrial fusion.

ROMO1 is a REDOX sensor that promotes mitochondrial fusion

Given the previous reports of ROMO1 as a regulator of ROS, we measure superoxide in our system. Superoxide concentrations increased 2-fold compared to control cells as measured by DHE staining (Fig 2A). Treatment of control cells with the complex III inhibitor antimycin A, which reduces efficiency of electron flux through the electron transport chain, led to a similar increase in ROS concentrations. Treatment of ROMO1 knockdown cells with antimycin A resulted in an additive 4.5-fold increase in superoxide over untreated control cells. We hypothesized that ROMO1 may couple ROS signaling to mitochondrial fusion and sought to identify potential redox-sensitive domains within ROMO1. Although ROMO1 was previously predicted to have a single membrane spanning domain [36, 37], our computational analyses comparing sequences of ROMO1 from metazoan and non-metazoans species revealed two putative transmembrane helices, as well

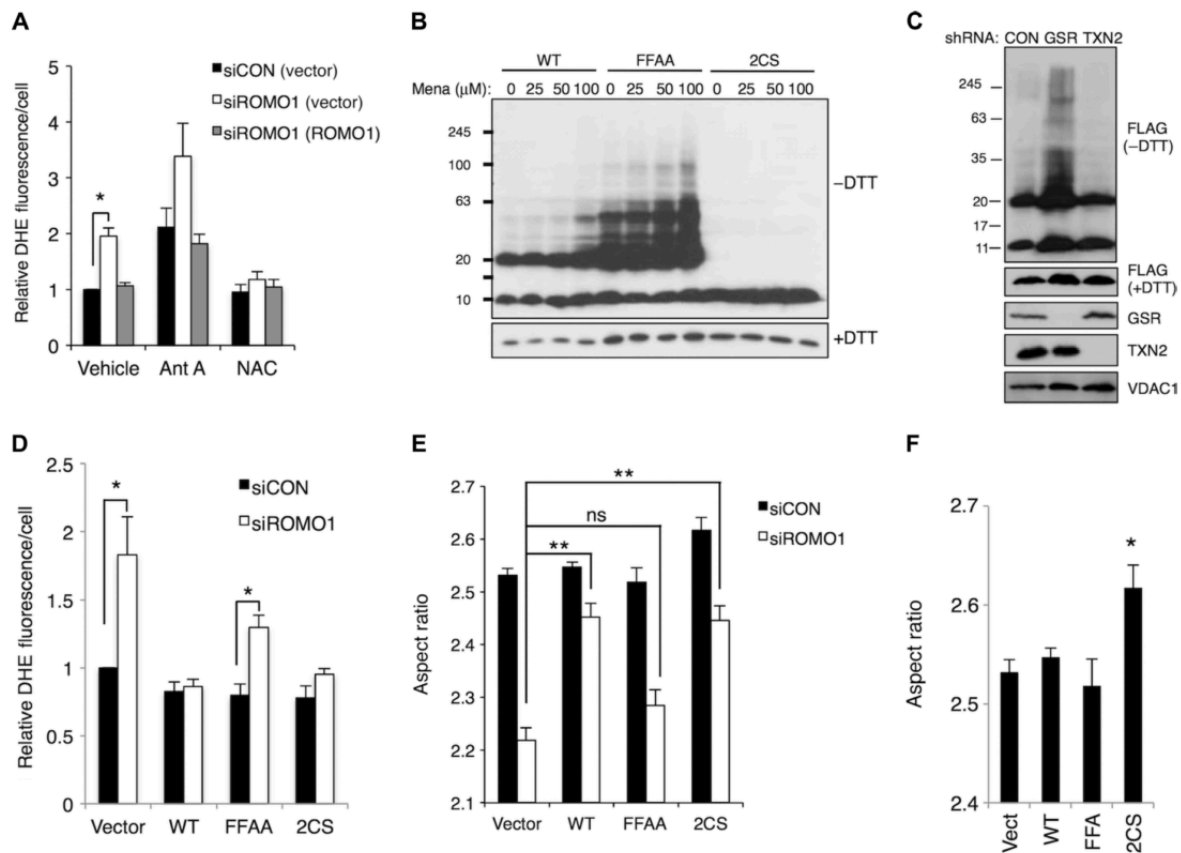


Fig 2: ROMO1 is a REDOX sensor that promotes mitochondrial fusion. A. DHE fluorescence emitted from cells transfected with control or ROMO1 siRNA with or without siResis-ROMO1. Effect of treatment with complex III inhibitor antimycin A (Ant A) and ROS scavenger *N*-acetyl cysteine (NAC) is shown. Error bars represent s.e.m of 3 independent experiments. Statistical significance determined by two-tailed unpaired *t*-test (* $p < 0.05$). B. Western blot of FLAG-ROMO1 WT, -FFAA, or -2CS mutants resolved on non-reducing (-DTT) and reducing gels (+DTT) following treatment of HEK293T cells with vehicle control (H₂O) or menadione in the presence of QVD, and VDAC loading control. N=3 independent biological replicates. C. Western blot of FLAG-ROMO1 resolved on non-reducing (-DTT) or reducing (+DTT) from control, GSR or TNX2 knockdown cells. N=3 independent biological replicates. D. Histogram of aspect ratio in control and ROMO1 knockdown cells expressing with either vector control or siRNA-resistant ROMO1 WT, FFAA or 2CS mutants. N=3 independent biological replicates. Statistical significance determined by two-tailed unpaired *t*-test (* $p < 0.05$). E. Histogram of aspect ratio in cells expressing vector control or siRNA-resistant ROMO1 WT, FFAA or 2CS mutants. N=3 independent biological replicates. Statistical significance determined by two-tailed unpaired *t*-test, ** $p < 0.01$). F. Histogram of aspect ratio in cells expressing vector control or siRNA-resistant ROMO1 WT, FFAA or 2CS mutants. N=3 independent biological replicates. Statistical significance determined by two-tailed unpaired *t*-test, * $p < 0.05$).

as two highly conserved cysteine residues that could serve as redox exchangers (Fig S2). Consistent with the latter possibility, FLAG-ROMO1 WT isolated from cells under steady-state conditions migrated as a single species of ~10 kD on reducing SDS-PAGE gels, but on non-reducing SDS-PAGE a substantial proportion of FLAG-ROMO1 resolved as a 20 kD species, with several additional oxidized species including some that were > 100 kD (Fig 2B). Treatment with menadione, which induces superoxide formation through redox cycling [41], substantially increased the slow-migrating oxidized ROMO1 species (Fig 2B), which disappeared upon removal of menadione (Fig S3A). Mutation of the highly conserved amino acids Phe⁶⁷ and Phe⁷⁰ to Ala (FFAA) appeared to increase the abundance of these higher molecular weight species; however, these species did not form when the two most highly conserved Cys residues, Cys¹⁵ and Cys⁷⁹ were mutated to Ser (ROMO1-2CS). These data indicate that ROMO1 may function as a redox switch serving to couple redox state in the inner membrane to cristae integrity. Redox switches are sensitive either to the glutathione or thioredoxin reducing systems [42]. To investigate this possibility, we knocked down glutathione reductase (GSR) or mitochondrial thioredoxin (TXN2) and observed a substantial increase in high molecular weight complexes containing ROMO1 in extracts from GSR-knockdown cells on non-reducing gels (Fig 2C). Steady-state ROS levels (Fig 2D) and normal morphology (Fig 2E, Fig S3B) was rescued in ROMO1 knockdown cells by expression of the non-oxidizable, monomeric ROMO1-2CS construct, but not of the heavily oxidized FFAA mutant. Furthermore, unlike the wild-type and FFAA constructs, ROMO1-2CS induced mild elongation of the mitochondrial network, despite accumulating to lower amounts (Fig 2F, Fig S3B-C). Taken together, these data indicate that ROMO1 is a redox-sensitive factor that forms inactive high molecular weight complexes in response to oxidative stress, and in its reduced, monomeric form drives mitochondrial fusion.

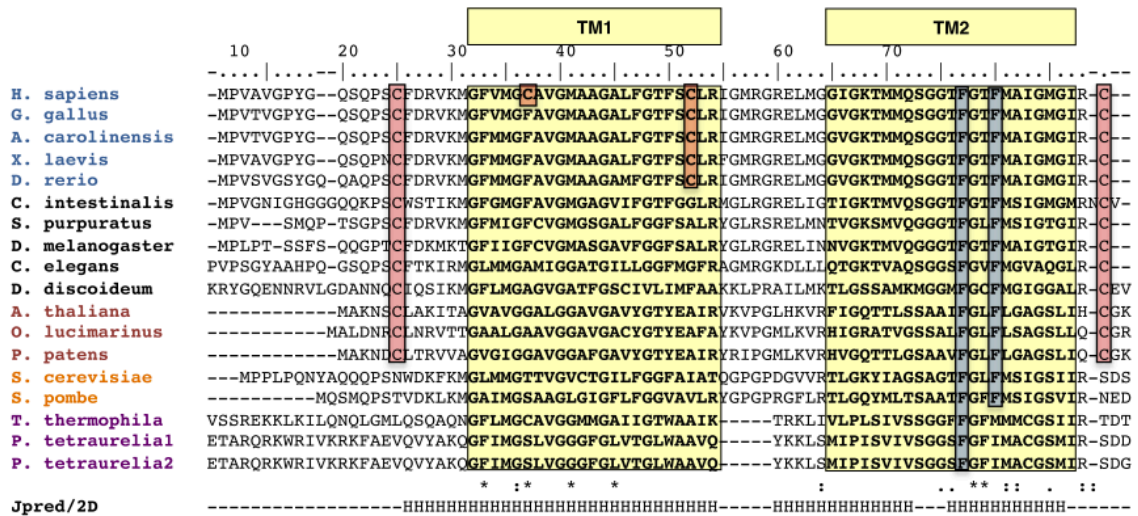


Fig S2: Full length multiple sequence alignment of ROMO1 with orthologs. Labels indicate the abbreviated species name. *Paramecium tetraurelia* has two copies (numbered here 1 and 2). Top lane: numbering of ROMO1 residues. Bottom lane: predicted helical structure by Jpred3 [66]. Yellow boxes: transmembrane helices (TM1, TM2) predicted by TMHMM [67]. The four Cys of ROMO1 and their conserved equivalents are marked in red boxes; Cys¹⁵ and Cys⁷⁹ are conserved in Metazoa, Amoebozoa (*Dyctiostelium discoideum*) and plants, but not in paramecium (Alveolata) or fungi. The two conserved Phe residues at 68 and 70, which are located in the C-terminal helix, are marked with blue boxes and are also conserved in fungi.

Fig S3: Reversible oxidation of ROMO1 and rescue of mitochondrial morphology with siRNA-resistant ROMO1 constructs. A. Western blot of FLAG-ROMO1 resolved on non-reducing (-DTT) and reducing gels (+DTT) following treatment of HEK293T cells with vehicle control (H₂O) or menadione in the presence of QVD for 8 hr with or without replacement with fresh medium for 20 hr. B. Top three rows: Mitochondrial morphology (TOMM20) and FLAG-ROMO1 staining in cells transfected with control siRNA (top 3 rows) or ROMO1 siRNA (bottom three rows). Middle row for each set shows higher magnification inset of TOMM20 staining. Cells were infected with lentivirus encoding an empty vector (Vector), FLAG-ROMO1 WT, -FFAA (Phe⁶⁷ and Phe⁷⁰ mutated to Ala), or -2CS (Cys¹⁵ and Cys⁷⁹ mutated to Ser). C. Western blots showing siRNA-resistant FLAG-ROMO1 proteins from B in cells transfected with control (CON) and ROMO1 siRNA.

ROMO1 is required for the integrity of mitochondrial cristae junctions

Western blot analysis suggested that the abundance of mitochondrial subcompartment marker proteins were unchanged in ROMO1 knockdown cells, suggesting that ROMO1 abundance does not affect mitochondrial mass or the abundance of outer membrane remodeling machinery (Fig 3A-B). As previous reports have localized ROMO1 to both outer and inner mitochondrial membranes [36, 43], we performed a protease protection assay using purified mitochondria (Fig 3C). In intact mitochondria, proteinase K treatment reduces the levels of the outer membrane protein TOMM20, but has no effect on ROMO1 or OPA1, which resides in the inner membrane and inner membrane space. In contrast, when proteinase K is added following an osmotic shock step that serves to rupture the outer mitochondrial membrane, both ROMO1 and OPA1 are degraded. While residence in the intermembrane space cannot formally be ruled out, when taken together with evidence for two transmembrane alpha helices in ROMO1, we propose that ROMO1 is an inner mitochondrial membrane protein with its N- and C-termini facing the IMS.

Analysis of inner mitochondria ultrastructure showed that ROMO1 knockdown cells had mitochondria with fewer or no cristae (Fig 3D-E). A small subset of mitochondria also displayed cristae stacks, suggesting loss of connectivity to the inner membrane, reminiscent of yeast mutants lacking the yeast ortholog of mitofilin, *fcj1* [44]. In support of these data, the ROMO1 yeast homolog Mgr2 has been identified by two studies that described a large protein complex known alternatively as mitochondrial inner membrane organizing system (MINOS) or mitochondrial organizing structure (MitOS) which establishes boundaries between cristae and juxtaposed

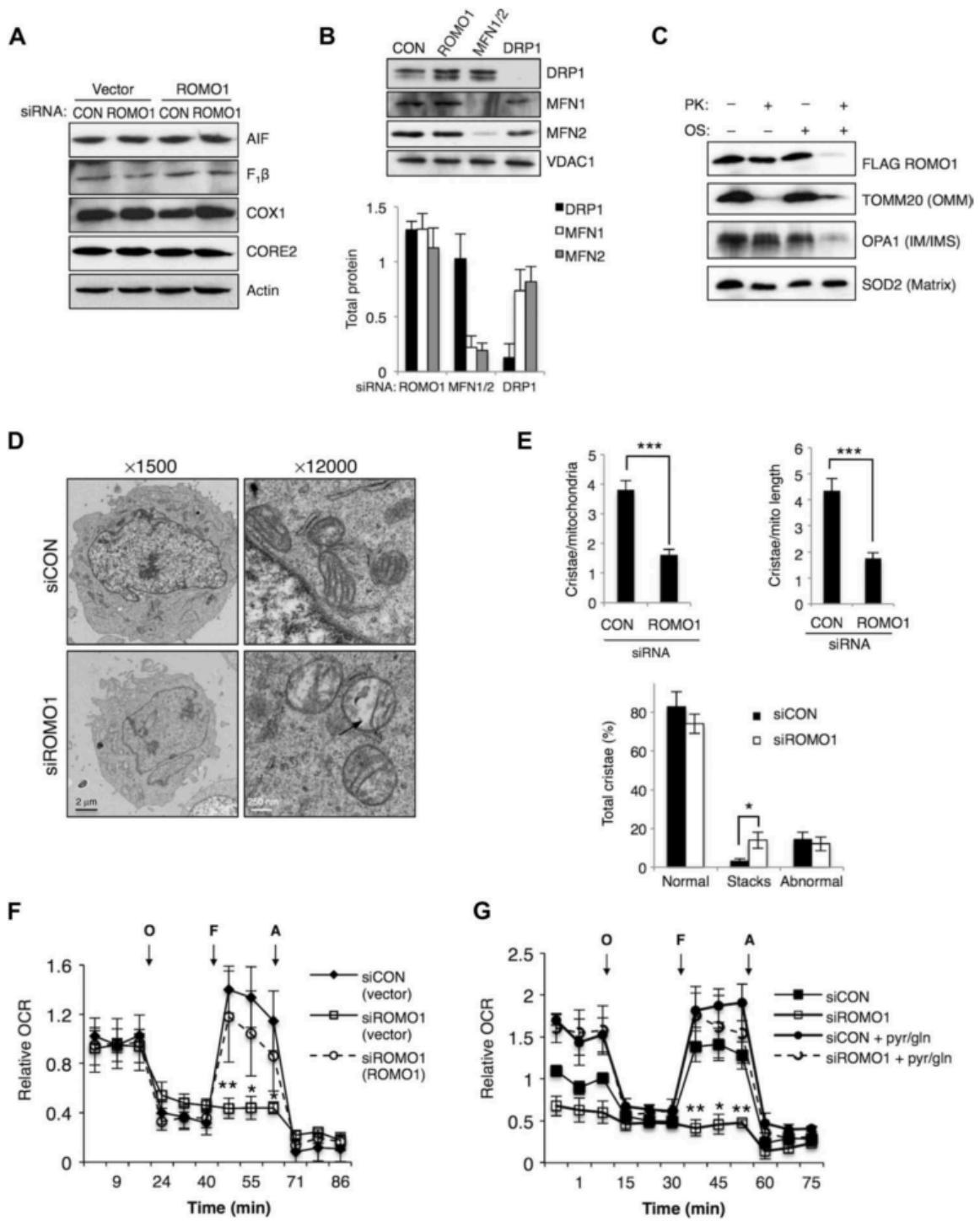


Fig 3: ROMO1 is required for the integrity of mitochondrial cristae junctions. A.

Western blot for mitochondrial markers AIF, ATP synthase F1 b subunit ($F_1\beta$), COX1, CORE2, and actin as the loading control. N=2 independent biological replicates. B. Top: Western blot for components of the outer membrane dynamics machinery (MFN1, MFN2 and DRP1) with VDAC1 loading control in cells transfected with siCON, siROMO1, siDRP1 or siMFN1 and siMFN2 together. Bottom: histogram showing quantitation of N=3 independent biological replicates. C. Western blot of a proteinase K (PK) protection assay on purified U2OS mitochondria before or after osmotic shock (OS) showing submitochondrial localization of FLAG-ROMO1. TOMM20 was used as a marker of the outer membrane, OPA1 was a marker of the inner membrane and inner membrane space, and SOD2 was used as a matrix marker. D. Electron micrographs of control or ROMO1 knockdown U2OS cells showing altered cristae in cells lacking ROMO1. Magnification = 1500X (left) and 12,000X (right). Scale bars indicated at bottom left of each image (left = 2 μ m; right = 250 nm). Images are representative of 2 independent experiments and 10 cells for a total of 100 mitochondria per condition. E. Histograms showing number of cristae per mitochondrion pixels in U2OS cells transfected with control or ROMO1 siRNA, (upper left panel, *** $p < 0.001$); the number of cristae per unit length of mitochondria in pixels (n=100 mitochondria) in U2OS cells transfected with control or ROMO1 siRNA, (upper right panel, *** $p < 0.001$); and the percentage of U2OS cells with cristae with normal, detached (stacks) or

abnormal appearance following transfection with control or ROMO1 siRNA (lower panel; * $p < 0.05$). Statistical significance determined by two-tailed, unpaired t -test. N=2 independent biological replicates. F. Oxygen consumption in control (solid black squares) or ROMO1 knockdown cells (empty squares) under basal conditions or following oligomycin (O), FCCP (F), and Antimycin A (A) treatment. ROMO1 rescue is shown in dashed line, empty circles. N=3 independent biological replicates. * $p < 0.05$, ** $p < 0.01$. Statistical significance determined by two-tailed, unpaired t -test. N=3 independent biological replicates. G. Oxygen consumption in control (solid marker) or ROMO1 knockdown (empty marker) cells in presence (circles) or absence of pyruvate and glutamine (pyr/gln) (squares), treated with oligomycin, FCCP, and Antimycin A as indicated. N=3 independent biological replicates. * $p < 0.05$, ** $p < 0.01$. Statistical significance determined by two-tailed, unpaired t -test. N=3 independent biological replicates.

boundary regions of the mitochondrial inner membrane [44, 45]. Exogenously expressed ROMO1 and mitofilin, a central component of MINOS, also formed a complex in mammalian cells (Fig S4), suggesting that ROMO1 localizes to cristae junctions [46]. However, silencing of ROMO1 did not affect the protein levels of the core MINOS components mitofilin or CHCHD3, nor does it alter the size of the complex as measured by gel fractionation, indicating loss of ROMO1 does not disrupt the integrity of the MINOS complex (Fig S4-C).

Despite these alterations, ATP concentrations were unchanged in both glycolytic and oxidative conditions, and mitochondria in cells lacking ROMO1 remain positive for MitoTracker Red and thus maintain potential (Fig S5). Oxygen consumption rates under basal conditions or following treatment with the ATP synthase inhibitor oligomycin were also unaffected (Fig 3F). In contrast, maximal (uncoupled) respiration elicited by FCCP treatment was abrogated in the absence of ROMO1, and was restored by reintroduction of siRNA-resistant ROMO1 (Fig 3F) or by adding pyruvate and glutamine to the assay medium (Fig 3G). Taken together, these data demonstrate that the cristae defects in ROMO1 knockdown cells do not interfere with normal function of the TCA cycle or with electron transport chain capacity but do reduce the maximal capacity of the respiratory chain.

ROMO1 is required for OPA1 isoform balance and oligomerization

As ROMO1 localizes to the inner mitochondrial membrane and its silencing and promotes fragmentation, we explored the effect of loss of ROMO1 on OPA1, the inner

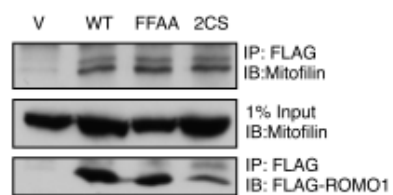
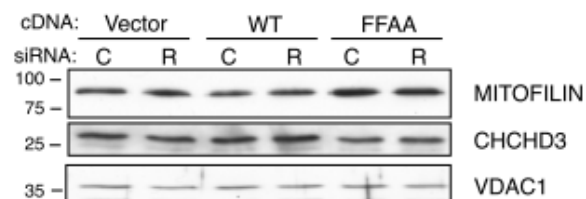
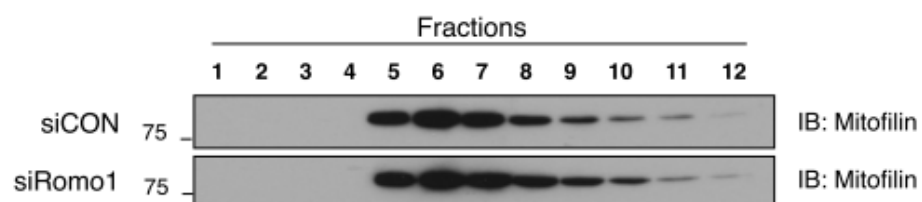
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Fig S4: ROMO1 associates with, but does not affect integrity of MINOS/MiTOS. A. Western blot showing co-IP of key MINOS component mitofilin with FLAG-ROMO1 WT, -FFAA, and -2CS along with mitofilin and FLAG-ROMO1 protein input blots. B. Western blot for MINOS components mitofilin and CHCHD3 in cells transfected with control (C) or ROMO1 (R) siRNA. Expression of ROMO1 WT or -FFAA constructs are shown. Vector = empty vector control. C. Mitofilin Western blots of extracts from control or ROMO1 knockdown cells following fractionation on Sepharose 4B.

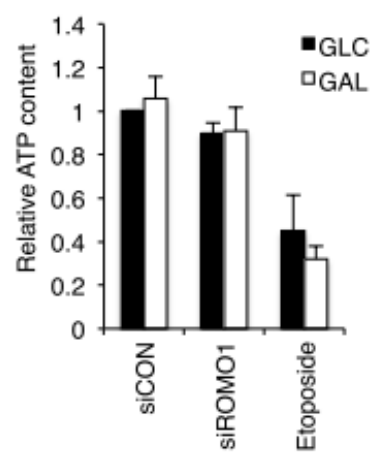
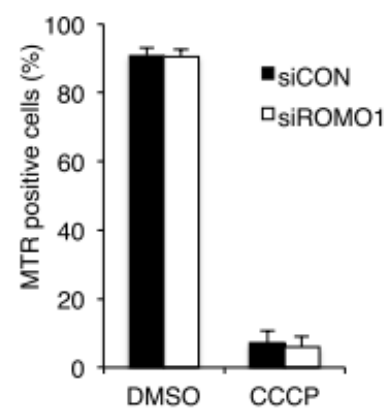
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Fig S5: Normal cellular ATP content and mitochondrial membrane potential in ROMO1 knockdown cells. A. Histogram showing ATP content in control and ROMO1 knockdown cells, as measured by relative luminescence. Experiment was performed in glycolytic (glucose cultured) and oxidative (galactose cultured) U2OS cells. Effect of treatment with the cytotoxic agent etoposide is shown. B. Histogram showing mitochondrial inner membrane potential revealed by mitotracker red fluorescence in control and ROMO1 knockdown cells. Effect of CCCP treatment is shown.

membrane fusion GTPase and regulator of cristae junction integrity. Immunoprecipitates of FLAG-ROMO1 WT, -FFAA, and -2Cs all contained long and short isoforms of OPA1 (Fig 4A and Fig S6A; [47-54]). We noted that silencing ROMO1 led to an imbalance in OPA1 isoforms that favored the accumulation of isoform C (Fig 4B), previously identified as a cleavage product of a transmembrane form of OPA1, denoted isoform 1 [55]. While normal isoform balance could be restored by reintroduction of siRNA-resistant FLAG-ROMO1-WT, expression of FLAG-ROMO1-FFAA caused the accumulation of long isoforms, suggestive of a gain-of-function (Fig S6B). We also observed accumulation of isoform c in wt MEFs in which ROMO1 was silenced (Fig 4C). Interestingly, silencing ROMO1 in OPA1^{-/-} MEFs expressing OPA1 long isoform, OPA1v1, led to the preferential accumulation of the processed form of OPA1, akin to what we observed with the endogenous protein in U2OS cells (Fig 4C). We attribute these effects to enhanced processing of OPA1 long isoform in cells lacking ROMO1.

Cells lacking ROMO1 are hypersensitive to apoptotic stimuli

These results prompted us to determine if the alteration in OPA1 isoform balance in cells lacking ROMO1 affected the oligomerization status of OPA1. Treatment of U2OS cells with a cell permeable crosslinker BMH results in the appearance on high molecular weight OPA1 oligomers (Fig 4D) [56]. However, the total amount of OPA1 protein present in oligomers was reduced from 35% in control cells to 5% in ROMO1 knockdown cells (Fig 4D). These data indicate that ROMO1 regulates cristae junction dynamics through OPA1 oligomerization. Cristae junction opening following tBID treatment is required during the

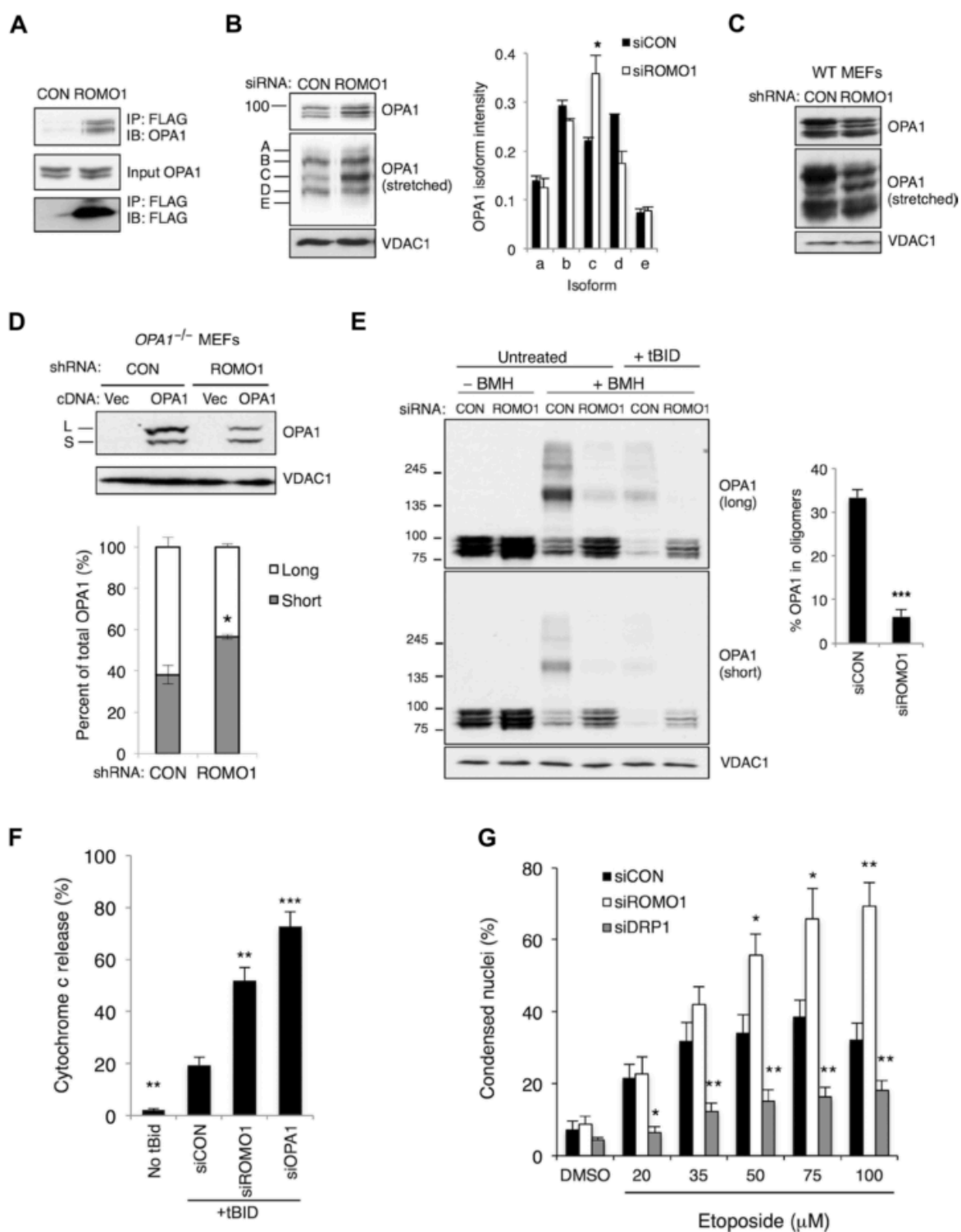


Fig 4: Loss of ROMO1 prevents OPA1 oligomerization and increases sensitivity to cell death. A. Western blot showing co-immunoprecipitation of FLAG-ROMO1 with OPA1. Representative of 3 independent biological replicates. B. Western blot showing relative abundance of OPA1 isoforms in control and ROMO1 knockdown cells with VDAC1 loading control. The vertical axis of the bottom OPA1 blot was stretched to enable visualization of the OPA1 isoforms (denoted A-E). Quantitation of isoform band intensity is shown at right. N=3 independent biological replicates, * $p < 0.05$. C. Left: Western blot showing effect of ROMO1 knockdown on OPA1 isoform balance in wt MEFs. Right, Top: Western blot showing amount of OPA1 long isoform v1 and its proteolytic cleavage product isoform c in OPA1^{-/-} MEFs stably reconstituted with OPA1v1 compared to vector control (Vec). Right, bottom: Quantitation of long and short isoform band intensity in control and ROMO1 knockdown cells. N=3 biological replicates, * $p < 0.05$. D. Left: Short and long exposures of Western blots showing formation of OPA1 oligomers and VDAC loading control following treatment of control or ROMO1 knockdown U2OS cells with cell-permeable cross-linker BMH. Right: Histogram showing % of total OPA1 band intensity present in oligomers (i.e. oligomer intensity/(oligomer+monomer intensity) X 100). N=3 biological replicates. *** $p < 0.001$. E. Histogram showing effect of ROMO1 knockdown on cytochrome c release after infection with Adeno-tBID. Knockdown of OPA1 shown as control. N=3 biological replicates, ** $p < 0.01$, *** $p < 0.001$. F. Histogram showing effect of increasing dose of etoposide on apoptosis in U2OS cells as quantified by condensed, apoptotic nuclear morphology in control (black bars), ROMO1 (white bars), or DRP1 (gray bars) knockdown cells. Error bars represent s.e.m. of 3 independent experiments, * $p < 0.05$, ** $p < 0.01$.

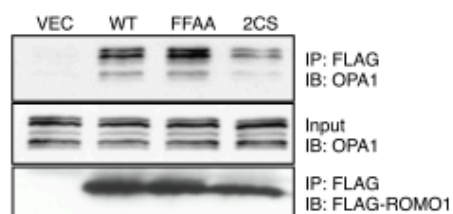
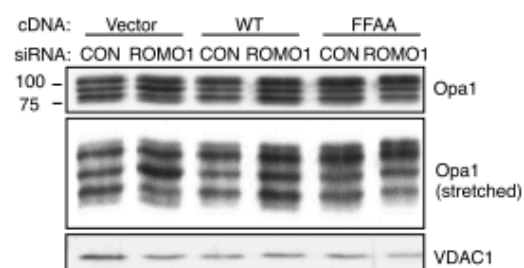
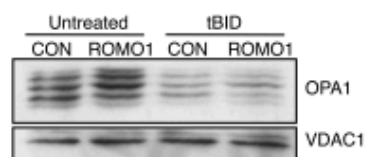
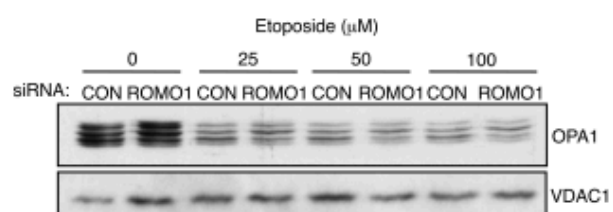
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Fig S6: Effect of ROMO1 status on OPA1 isoform balance. A. Western blot showing coimmunoprecipitation of OPA1 with FLAG-ROMO1 WT, -FFAA, and -2CS mutants. B. Western blot showing accumulation of OPA1 isoform c in ROMO1 knockdown cells (ROMO1) can be rescued by reintroduction of siRNA-resistant ROMO1 WT but not -FFAA. C. Western blot showing loss of OPA1 protein in both control siRNA (CON) and ROMO1 siRNA (ROMO1) transfected cells following infection with Adeno-tBID. D. Western blot showing loss of OPA1 protein in cell treated with etoposide at indicated concentrations. In all cases, accumulation of OPA1 isoform c can be seen in ROMO1 knockdown cells.

early stages of apoptosis for cytochrome *c* release [56], processes that are associated with the loss of OPA1 oligomers and subsequent remodelling of the inner mitochondrial membrane. We evaluated the effect of ROMO1 knockdown on tBID-induced cytochrome C release, and as previously shown, knockdown of OPA1 enhanced cytochrome *c* release due to opening of cristae and fragmentation of the outer membrane [57]. Following tBID treatment, cytochrome *c* release was enhanced 2.5-fold in cells lacking ROMO1, consistent with the loss of OPA1 oligomers observed at steady state (Fig 4E). This sensitization to cytochrome *c* release in ROMO1 knockdown cells correlated with an increase in cell death in response to treatment with the chemotherapeutic drug etoposide (Fig 4F). The sensitization to cell death was accompanied by the loss of OPA1 protein (both oligomers and monomers), but no change in isoform balance was seen (Fig S6C-D). Taken together, we conclude that ROMO1 controls the generation of OPA1 isoforms, and that OPA1 is a downstream effector of ROMO1 function.

Discussion

Here we report a genome wide screen in mammalian cells to identify novel regulators of mitochondrial dynamics using an unbiased quantitative analysis of mitochondrial morphology. We focused on the role of ROMO1, silencing of which leads to fragmentation of the mitochondrial network due to a defect in mitochondrial fusion. ROMO1 has been shown to regulate the generation of ROS within cells, linking it to cell fate decisions like proliferation, senescence and cell death [35, 36, 38, 39]. However, the molecular explanation for this has been unclear. We show that loss of ROMO1 increases ROS and that

ROMO1 itself forms disulfide bridges, and incorporates into higher molecular weight species in response to oxidative stress processes, which are counteracted by the glutathione system.

How does reduced ROMO1 regulate mitochondrial morphology? We envision that formation of disulfide bridges within ROMO1 in response to oxidative stress is inhibitory, resulting in a fragmented mitochondrial network allowing for containment of oxidative damage and removal of damaged mitochondria. The reversibility of ROMO1 oxidation states appears critical for its function, as expression of the non-oxidizable ROMO1-2CS mutant drives mitochondrial elongation, suggesting that ROMO1 is active in its monomeric, reduced state. Consistent with this idea, ROMO1-FFAA, which is more highly oxidized under steady-state conditions, is unable to support mitochondrial fusion. Our computational analysis of ROMO1 orthologs in primordial species provides mechanistic insight as to how ROMO1 can be modulated by oxidative stress. We propose that with two transmembrane helices, ROMO1 would adopt an architecture in which the most highly conserved Cys residues in metazoans would be juxtaposed in the same mitochondrial subcompartment, thus permitting the formation of both homotypic and/or heterotypic Cys-Cys bridges. Interestingly, these Cys residues are absent in yeast, suggesting that Mgr2/ROMO1 may have obtained the capacity to sense oxidative stress in the IMS or matrix as an additional mode of regulation of OPA1, CJs, and IM fusion in higher organisms.

We provide the first evidence that ROMO1 is essential for the integrity of mitochondrial cristae, supported both by ultrastructural analysis and functionally by the reduced threshold

for cytochrome *c* release in cells lacking ROMO1. In the absence of ROMO1, mitochondria either lack cristae or have a minor population of detached cristae stacks, reminiscent of cristae in MINOS mutant yeast strains [58]. Consistent with this observation, mitochondria without ROMO1 display impaired respiration during uncoupling. We attribute these effects to defective compartmentalization of fuel and of ETC components in cristae invaginations in the absence of ROMO1, resulting in a less efficient electron transport chain with the subsequent increase in ROS production we observed.

Several lines of evidence point to a pathway in which ROMO1 couples mitochondrial ROS to OPA1 oligomerization and cristae junction integrity. First, in the absence of ROMO1, OPA1 isoform balance is changed, likely due to altered processing. Perhaps as a consequence of this disruption in OPA1 processing, the formation or maintenance of oligomers of OPA1 is lost upon ROMO1 silencing. In the absence of ROMO1, OPA1 oligomerization is reduced to levels seen in apoptotic cells which underlies the enhanced sensitivity to apoptotic insults. Second, ROMO1 and OPA1 reside in the inner mitochondrial membrane and form a precipitable complex in cells. Third, silencing or ablation of ROMO1 or OPA1 phenocopy each other, with respect to cristate junction integrity and inner membrane fusion [59]. Lastly, loss of both ROMO1 and OPA1 sensitizes cells to cytochrome *c* release and cell death [57]. Taken together, our work has also provided new insights into how changes in mitochondrial redox state, often examined in the context of cell stress, may lead to the opening of cristae junctions and fragmentation of the mitochondrial network [60, 61]. Recent evidence suggests that OPA1 regulates cristae dynamics in response to changes in mitochondrial redox state upon inhibition of the ETC

[62], which we propose involves ROMO1. Impaired mitochondrial fission contributes to chemotherapy resistance [63]. ROMO1 is upregulated in a subset of human tumours [64, 65] (www.cbioportal.org), consistent with the concept that cancer cells are resistant to apoptosis. We propose that targeting ROMO1 in an effort to open the cristae in drug-resistant cancer cells may be of therapeutic benefit.

Materials and Methods

Antibodies and Reagents

TOMM20 (Santa Cruz), F1B, Core2 and Cox1 (all from Mitosciences), FLAG M2 (Sigma), Mitofilin (Abcam), CHCHD3 (Abcam), VDAC1 (Cell Signaling), Opa1 (BD Transduction Labs), Opa1 (Abcam), Cytochrome C (BD Pharmingen), AIF (Santa Cruz). Lipofectamine RNAiMAX, Hoechst and Mitotracker Red and dihydroethidium were all from Invitrogen. Polyethylenimine, CCCP, Antimycin A, N-Acetylcysteine, Sepharose 4B, and Etoposide were all from Sigma. Oligomycin and FCCP were from Seahorse Bioscience. Caspase inhibitor QVD was from Calbiochem. Halt Protease Inhibitor Single-Use Cocktail was from Thermo Scientific. BMH crosslinker was from Fisher Scientific.

Imaging Screen and Candidate Validation

Pools of four siRNA SMARTpool duplexes targeting 18,255 human genes (Dharmacon) were spotted into 384 well plates at 10 nM and reverse transfected into 450 HeLa cells (P34-36) using 0.025 uL of Lipofectamine RNAiMAX (Invitrogen). 72 hr later, cells were fixed and stained with anti-TOMM20 antibody and 1 ug/mL Hoechst to visualize mitochondria

and cell nuclei. 8 fields per well were acquired using an automatic Cellomics Vti microscope equipped with a 40X objective. Robust Z-scores were calculated as $[x - \text{median}(\text{sample})]/[\text{median absolute deviation} \times 1.4826]$. The algorithm to detect mitochondrial network length uses TOMM20 staining signal and negates perinuclear signal to avoid condensation artifacts. The average length:width (aspect ratio) of measurements across multiple fields was used to quantify mitochondrial morphology on a population basis. We applied the MEAN_ObjectEqEllipseLWRCh1 metric within the Morphology Bioapplication provided with the Cellomics vHCS Scan image analysis software to determine the aspect ratio of objects stained positive for TOMM20. After algorithm analysis, the efficacy of deconvoluted siRNA pools was then determined for candidates for which images from 10 wells were verified manually to display an elongated or fragmented state. Analysis of three datasets revealed a mean Pearson correlation coefficient of 0.82, demonstrating high plate-to-plate reproducibility.

***In vitro* Fusion assay**

HeLa cell lines stably overexpressing the N-MitoVZL or C-MitoLZV markers were silenced for Romo1 (D-015268-04, Dharmacon) or with control siRNA (D-001810-01, Dharmacon). For each cell line, five 15cm tissue culture plates were seeded at 25000 cells/cm² and silenced with 20nM siRNA and Dharmafect I, according to the manufacturer's protocol. Following four days of silencing, cells were harvested and mitochondria were isolated by differential centrifugation and stored at -80C as previously described [40]. Mitochondrial fusion assays were performed with a total of 100 ug of mitochondria as previously described [25]. Briefly, mitochondria were pelleted and incubated at 4C for 30 minutes to promote

docking. Following resuspension, mitochondria were incubated at 37C for 10 minutes to allow fusion. Next, mitochondria were treated with trypsin for 10 minutes at 4C to remove protein from broken mitochondria, and the trypsin was inhibited with SBTI. Finally, mitochondria were lysed and luciferase activity measured using the Renilla Luciferase Assay System (Promega, WI, USA) and Glomax Luminometer (Promega, WI, USA), according to the manufacturer's protocol.

Plasmid Constructs

The human ROMO1 ORF was cloned into pLenti6 destination vector (Invitrogen) and tagged at the 5' end with a FLAG-tag. siRNA-resistance of FLAG-ROMO1, as well as FFAA and 4CS mutations were generated by quickchange mutagenesis.

Immunoprecipitation, SDS-PAGE and Western Blotting

HEK293T cells were transfected using polyethylenimine (SIGMA). 40 h later, cells were collected in PBS, pelleted and processed as described [66].

Protease Protection Assay

U2OS cells from five 95% confluent 15 cm dishes were trypsinized and pelleted. Cells were washed with PBS and then resuspended in mitochondrial isolation buffer (MIB) + protease inhibitor cocktail: 200 mM Mannitol, 68 mM sucrose, 10 mM Hepes-KOH pH7.4, 10 mM

KCl, 1 mM EDTA pH 8.0 in KOH, 1 mM EGTA pH 8.0 in KOH, 0.1% BSA. Resuspended cells were incubated on ice for 30min and then lysed by passing cells through needles as follows: 2 x 25 gauge, 3 x 27 gauge, 35 x 30 gauge. Lysis was stopped once cells reached 70% trypan-blue positivity. Lysate was centrifuged at 600g for 10 min to clear unlysed cells and debris. The supernatant was centrifuged at 600g for 10 min and then at 5 500g for 15 min to obtain the heavy membrane fraction. Heavy membrane pellet was resuspended in 400 μ L MIB and then divided equally into 4 tubes treated as follows: (Tube 1: untreated; Tube 2: 20 μ g Proteinase K; Tube 3: Osmotic shock (700 μ L 20mM KCl added to 100 μ L of mitochondria); Tube 4 Osmotic shock + 80 μ g Proteinase K). Reactions were incubated for 75 min and then centrifuged at 15 000g for 15 min. Mitochondrial pellet was resuspended in 100 μ L MIB + 5 μ L 100% TCA to precipitate protein. Protein lysates were analysed by SDS-PAGE and Western Blotting.

Electron microscopy

In a 6-well dish, 125,000 cells were reverse transfected with siRNA, washed 72 h later with PBS, pelleted and resuspended in 2% glutaraldehyde/PBS for embedding as described [67].

Mitochondrial stress test

Cells were reverse transfected on Seahorse XF24 cell culture microplate on day 0. Where applicable, lentivirus was added to wells 4 hr later. On day 3, cells were washed 3X with 500 μ L of Seahorse XF assay medium and oxygen consumption measured using oligomycin,

FCCP and Antimycin A (all at 0.5 μ M) as controls. Protein was quantified using BCA (Thermo Scientific) for normalization.

Immunofluorescence

Cells seeded in 384-well imaging plates were stained as described [68] and imaged with a 60X water objective (NA = 1.2) on an Olympus Fluoview FV1000 Confocal Laser Scanning Microscope.

ROS Measurements

72h after siRNA transfection, cells were stained with 20 μ M DHE for 30 min and fluorescence was analyzed using the Olympus Fluoview FV1000 confocal laser scanning microscope. Total fluorescence intensity was normalized to cell number to report relative DHE fluorescence. Where indicated, cells were pretreated with 10 mM N-acetyl cysteine for 4h or 20 μ M Antimycin A for 1h.

ATP measurements

Cellular ATP content was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega) using a BioTek Synergy2 plate reader. Values were normalized to cell number.

Gel chromatography

Cells lysed with 500 uL CoIP Buffer containing 1% TX-100 and protease inhibitors without DTT, sonicated, and centrifuged at 13 000 rpm for 20 min. Supernatants were applied to a Sepharose 4B column and 250 uL fractions were collected every 8 min for SDS-PAGE and Western Blotting analysis.

Cytochrome *c* release

Cells were infected with adenovirus expressing a TET-inducible tBid (gift from G. Shore, McGill University) with 1ug/mL doxycycline and 12.5 mM QVD and stained with cytochrome *c* and TOMM20 antibodies prior to imaging on the Cellomics Arrayscan VTi. Cytochrome *c* release from TOMM20-positive structures was quantified using a colocalization algorithm.

Condensed Nuclei death assay

Cells were treated with etoposide for 20 hr, fixed and stained with 10 ug/mL Hoechst, then imaged using an OPERA confocal imager (Perkin Elmer). Condensed nuclei were quantified using an algorithm based on nuclear size and intensity of Hoechst staining.

Statistical Analyses

Adjustment for multiple testing in ANOVA models was performed using R version 3.0.1 with the multcomp and sandwich packages [69-71]. All other p values were determined using a student's unpaired, two-tailed *t*-test.

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Appendix 3

Curriculum Vitae

CURRICULUM VITAE

Andy Cheuk-Him Ng

Languages: English, Cantonese

Education

1. MD/PhD 2010-2017 – ongoing
University of Ottawa, Ottawa, Canada
2. Honours B.Sc. with Specialization in Biochemistry CO-OP - 2003-2008
Summa Cum Laude - cGPA: 9.3/10
University of Ottawa, Ottawa, Canada

Awards

1. James M. Inglis Fellowship for Mental Health Studies (2013, 2014); \$5000 each
2. Charles Sean Alger Memorial Scholarship (2014); \$5000
2. CIHR - Frederick Banting and Charles Best Canada Graduate Scholarship (2012 – 2015); \$30000/yr
3. MS.c Ph.D Biochemistry Poster Day Award (2010, 2013)
4. Ph.D. Admissions Scholarship (2012 – 2015); \$9000/yr
5. Faculty of Medicine Priorities – Student Aid (2010 – 2011, 2011 - 2012)
6. Fisher Scientific Awards of Excellence in Graduate Studies – M.Sc. (2009 –2010)
7. Faculty of Medicine Graduate Studies Master's Seminar Day Prize (2010)
8. NSERC – Canada Graduate Scholarships (CGS) Master's (2008 – 2010)
9. University of Ottawa Excellence Scholarship (2008 – ongoing)
10. NSERC – Undergraduate Student Research Awards (2005, 2007)
11. Dean's Honour List (2003 – 2008)
12. University of Ottawa Admissions Scholarship (2003 – 2008)

Experience

1. Sept 2010 – to present *Medical Student / Graduate Student*
M.D/Ph.D Programme
Faculty of Medicine,
University of Ottawa, Ottawa, ON, CANADA.
Supervisor: Dr. R.A. Screatton
2. Sept 2008 – August 2010 *Graduate Student*
Children's Hospital of Eastern Ontario Research
Institute
Department of Cellular and Molecular Medicine,
Faculty of Medicine,
University of Ottawa, Ottawa, ON, CANADA.
Supervisor: Dr. R.A. Screatton

3. Sept 2006 – Dec 2006 *CO-OP,*
 May 2007 – Aug 2007 *CO-OP/NSERC Summer Student,*
 Sept 2007 – April 2008 *Honours Student, and*
 May 2008 – Aug 2008 *Summer Student*
 Children's Hospital of Eastern Ontario Research
 Institute
 Department of Cellular and Molecular Medicine,
 Faculty of Medicine,
 University of Ottawa, Ottawa, ON, CANADA.
 Supervisor: Dr. R.A. Screatton

4. May – Aug 2006 *CO-OP Research Student*
 Department of Cellular and Molecular Medicine,
 Faculty of Medicine,
 University of Ottawa, Ottawa, ON, CANADA.
 Supervisor: Dr. J.K. Ngsee

5. May – Aug 2005 *NSERC Summer Research Student*
 Department of Biology, Faculty of Science,
 University of Ottawa, Ottawa, ON, CANADA.
 Supervisor: Dr. C. Charest

Publications

1. Sawyer, S.L.*, **Ng, A. C-H.***, Innes, A.M., Wagner, J.D., Dymment, D.A., Tetreault, M., Care4Rare Canada Consortium, Majewski, J, Boycott, K.M., Sreaton, R.A.*, Nicholson, G*. Homozygous mutations in MFN2 cause multiple symmetric lipomatosis associated with neuropathy. Human Molecular Genetics, 24(18): 5109-14, (2015).

*equal contribution

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3. **Ng, A. C-H.**, Baird, S.D., Sreaton, R.A. Essential Role of TID1 in maintaining mitochondrial membrane potential homogeneity and mitochondrial DNA integrity. Molecular and Cellular Biology, 34(8):1427-37, (2014).
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9. Ng, L.K., **Ng, A.**, Cholette, F., Davis, C., Optimization of recovery of human DNA from envelope flaps using DNA IQ System for STR genotyping. Forensic Sci Int Genet, 1 (3-4), 283-6 (2007).

Abstracts

1. **Ng, A. C-H.** Baird, S.D., Sreaton, R.A. Essential Role of TID1 In Maintaining Mitochondrial Membrane Potential Homogeneity And Mitochondrial DNA Integrity. Keystone Symposia Conference Q5: Mitochondrial Dynamics and Physiology. Santa Fe, United States of America. February 2014.
2. **Ng, A. C-H.** Baird, S.D., Sreaton, R.A. Essential Role of TID1 In Maintaining Mitochondrial Membrane Potential Homogeneity And Mitochondrial DNA Integrity. CHEO Research Day (2013).
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*Both authors contributed equally.
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Teaching Experience

1. Laboratory Demonstrator, BCH 2333 Introduction to Biochemistry, Winter 2009 (6 hrs per session/2 sessions per week)
2. Co-supervision of a 4th year BCH Honours project. Student: Ariane Dumoulin May 2008 – April 2010.
3. Piano Teacher 2001 – 2011.