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Regulation Of Mitochondrial Dynamics And Quality Control In Mammalian Cells

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Regulation Of Mitochondrial Dynamics And Quality Control In Mammalian Cells

Abstract
Mitochondria are conserved eukaryotic organelles that carry out myriad cellular functions including energy generation, reactive oxygen species signaling, and lipid synthesis. In metazoans, mitochondria form dense, reticular networks that must be maintained through rigorous quality control mechanisms. Here, we describe three distinct aspects of mitochondria network homeostasis. In chapter 3, we investigate the spatiotemporal dynamics of mitophagy, tracking the association of autophagy machinery with individual damaged mitochondria. Using a range of damage paradigms, we dissect the kinetics of mitochondrial turnover and demonstrate that ALS-linked mutations in the proteins Optineurin and TBK1 interfere with efficient mitophagy. In chapter 5, we characterize a mechanism by which the actin cytoskeleton regulates mitochondrial network dynamics in interphase cells. Specifically, we investigate a traveling wave of actin filaments propagating through interphase mitochondria networks at a rate of ~5µm/min. This actin wave associates with 20% of mitochondria at a given time, where it transiently promotes mitochondrial fission. After 3-5 minutes, the actin wave travels to a neighboring region of the mitochondrial network, and the fragmented mitochondria fuse back together. We hypothesize that cycling actin waves function as constitutive regulators of mitochondrial length and prevent mitochondrial hyperfusion which is linked to cell senescence. Finally, in chapter 6, we identify a mechanism of mitochondrial network inheritance in somatic cells. Using spinning disk confocal and lattice light-sheet microscopy, we determine that mitochondria are highly dynamic through mitosis. We find that this motility is not dependent on microtubules, but rather on the actin cytoskeleton. Specifically, we identify a traveling wave of sub-cortical actin filaments propagating through metaphase mitochondria networks at ~15 µm/min and promoting bursts of mitochondrial motility. Inhibition of actin polymerization promotes aggregation of metaphase mitochondria networks and interferes with mixing of mtDNA nucleoids prior to division. Together these results provide a detailed picture of mitochondria network maintenance throughout the life cycle of mammalian cell lines.

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REGULATION OF MITOCHONDRIAL DYNAMICS AND QUALITY CONTROL IN
MAMMALIAN CELLS

Andrew Stephen Moore
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in
Neuroscience
Presented to the Faculties of the University of Pennsylvania
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To my father, Malcolm A.S. Moore, whose passion and curiosity are a constant source of inspiration.
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Mitochondria are conserved eukaryotic organelles that carry out myriad cellular functions including energy generation, reactive oxygen species signaling, and lipid synthesis. In metazoans, mitochondria form dense, reticular networks that must be maintained through rigorous quality control mechanisms. Here, we describe three distinct aspects of mitochondria network homeostasis. In chapter 3, we investigate the spatiotemporal dynamics of mitophagy, tracking the association of autophagy machinery with individual damaged mitochondria. Using a range of damage paradigms, we dissect the kinetics of mitochondrial turnover and demonstrate that ALS-linked mutations in the proteins Optineurin and TBK1 interfere with efficient mitophagy. In chapter 5, we characterize a mechanism by which the actin cytoskeleton regulates mitochondrial network dynamics in interphase cells. Specifically, we investigate a traveling wave of actin filaments propagating through interphase mitochondria networks at a rate of ~5µm/min. This actin wave associates with 20% of mitochondria at a given time, where it transiently promotes mitochondrial fission. After 3-5 minutes, the actin wave travels to a neighboring region of the mitochondrial network, and the fragmented mitochondria fuse back together. We hypothesize that cycling actin waves function as constitutive regulators of mitochondrial length and prevent mitochondrial hyperfusion which is linked to cell senescence. Finally, in chapter 6, we identify a mechanism of mitochondrial network inheritance in somatic cells. Using spinning disk confocal and lattice light-sheet microscopy, we determine that mitochondria are highly dynamic through mitosis. We find that this motility is not dependent on microtubules, but rather on the actin cytoskeleton. Specifically, we identify a traveling wave of sub-cortical actin filaments propagating through metaphase mitochondria networks at ~15 µm/min and promoting bursts of mitochondrial motility. Inhibition of actin polymerization promotes aggregation of metaphase mitochondria networks and interferes with mixing of mtDNA nucleoids prior to division. Together these results provide a detailed picture of mitochondria network maintenance throughout the life cycle of mammalian cell lines.
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CHAPTER 1: INTRODUCTION

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1.1 Thesis overview

In this dissertation, I will present my work studying three distinct aspects of mitochondrial network maintenance: (1) the turnover of aged or damaged mitochondria by autophagy (Chapter 2-4), (2) The regulation of mitochondria morphology by dynamic fission and fusion (Chapter 5), and (3) the mechanism coordinating mitochondrial network inheritance upon somatic cell division (Chapter 6).

In the introduction, I will present a brief historical overview of mitochondria research and describe several key mitochondrial functions in mammalian cells.
1.2 Mitochondria: a historical overview

Mitochondria are highly abundant organelles that are found in all eukaryotes. These double membrane structures support the energetic needs of complex cells and have been hypothesized to be the catalyst for multicellular evolution (Lane, 2017). While mitochondria are structurally and functionally heterogenous, the archetypal mitochondrion is composed of four major compartments: outer membrane, inner membrane, intermembrane space, and matrix. The outer mitochondrial membrane (OMM) is porous, allowing molecules <5kDa to pass into the intermembrane space (IMS). In contrast, the inner mitochondrial membrane (IMM) is highly impermeant and densely packed with electron transport chain (ETC) complexes. The IMM forms intricate folds known as cristae, which increase the membrane surface area in order to maximize the space for oxidative phosphorylation. Within the inner membrane is the mitochondrial matrix, which contains mitochondrial DNA, specialized mitochondrial ribosomes, key metabolites, and houses the Krebs cycle which generates ATP, amino acids, and reducing agents required for oxidative phosphorylation. Our detailed knowledge of mitochondria structure and function has developed over ~125 years. Here, I will review some of the major milestones in mitochondrial research, with a special focus on those achieved through the use of microscopy.

1.2.1 Subcellular granules

In the late 19th century, the development of novel histological stains and fixation techniques led to a revolution in biological microscopy, and, for the first time, offered microscopists a window into the previously invisible world of subcellular organization. In 1890, University of Leipzig professor Robert Altmann published “the elementary organisms and their relationships to the cell,” in which he identified granular structures that he termed “bioblasts.” Altmann noted – quite shrewdly as history would prove – that
bioblasts bore a striking resemblance to bacterial cells; an observation that would eventually come to form the basis of the now widely accepted endosymbiotic theory (Altmann, 1890; Cowdry, 1953; Sagan, 1967). Contemporaneous with Altmann’s characterization of bioblasts, other groups reported the discovery of subcellular structures, including plasmafaden, microsomen, paramiton, and chondrioconten (Lewis & Lewis, 1915). As it turned out, these different structures were in fact identical, but slow communication, non-standardized fixation/staining protocols, and the lack of standard nomenclature led to widespread misclassification. By 1899, the German anatomist Carl Benda coined the term mitochondrion, from the Greek “mitos,” meaning thread, and “chondrion,” meaning granule (Lewis & Lewis, 1915). Eventually “mitochondrion” would be adopted as the official nomenclature, but the precise function of these microscopic granules would remain elusive for several decades.

1.2.2 Live imaging of mitochondria

Early in the 20th century, novel tissue culture techniques allowed for the isolation and maintenance of animal cells on glass dishes (in vitro). Using this methodology, researchers could, for the first time, track the dynamics of living mitochondria by transmitted light microscopy. It became immediately clear that mitochondria were not static grain-like structures as they appeared in fixed tissues, but rather highly dynamic shape-shifters. A classic text from the husband and wife team of Lewis and Lewis described mitochondrial dynamics in cultured embryonic chick cells:

The mitochondria are slightly refractive bodies which vary greatly in shape, size, and position. In the living cell these bodies are never quiet, but are continually changing in shape, size, and position. Often as many as fifteen or twenty shapes may be exhibited by a single mitochondrion within as many minutes. This extreme plasticity of the mitochondria is a very important characteristic and was shown in
every preparation examined. It is certainly a feature which must be reckoned with in any attempt to classify or to analyze their behavior from fixed material.

(Lewis & Lewis, 1915)

The text includes meticulously drawn mitochondria (Fig. 1.1) and systematically categorizes the effects of chemicals, temperature, and cell cycle stage on mitochondria network morphology and dynamics. Further, Lewis & Lewis identify "degenerate mitochondria" and describe what is perhaps the first account of selective mitochondrial autophagy (mitophagy). It would not be for another 75 years until the field would begin to provide mechanistic explanation for many of the observations that the Lewises first made.
1.2.3 Mitochondria structure and function

Starting with Otto Warburg in 1913, several groups tried unsuccessfully to isolate pure mitochondria by cell fractionation techniques (Ernster and Schatz, 1981). In 1934, Bensley and Hoerr used a novel centrifugation protocol and succeeded in isolating mitochondria from guinea pig liver (Bensley & Hoerr, 1934). In vitro analyses of mitochondrial fractions shed light on the chemical makeup of mitochondria and provided clues as to their metabolic functions. By the middle of the 20th century, it was quite clear that mitochondria functioned as the central energy generators of the cell (Palade, 1952). Further insight into mitochondrial structure came with the development of electron microscopy. The first transmission electron microscope (TEM) was constructed by Ernst Ruska in 1931. These instruments had significantly greater resolving power than transmitted light microscopes and revealed ultrastructural details of mitochondria morphology that were previously unknown (Claude & Fullam, 1945). George Palade, considered among the most influential cell biologists in history, used electron microscopy to uncover the internal organization of mitochondria, describing an ~8nm outer membrane, internal cristae which appeared as layered ridges, and a central matrix compartment (Palade, 1953).

1.2.4 Fluorescence

The advent of fluorescence microscopy ushered in a golden age of cell biological research. Using modern epifluorescence microscopes equipped with high numerical aperture (NA) objective lenses and sensitive digital cameras, researchers could acquire high-resolution images and movies of living cells. Starting in the early 1980s, a number of fluorescent probes to label mitochondria were developed.
1.2.4.1 Cationic dyes

In 1980, a cell permeant, cationic fluorescent dye called rhodamine 123 was found to efficiently label mitochondria (Johnson et al., 1980). Unlike the previous standard for mitochondrial labeling, Janus Green B, rhodamine 123 did not result in robust cytotoxic effects (Johnson et al., 1980) allowing longer term tracking of mitochondrial dynamics. Rhodamine 123 also had the desirable characteristic of membrane potential dependence: its fluorescence intensity could be used to quantitively estimate mitochondrial polarization state (Johnson et al., 1981). In the following years, a number of other sensitive cationic dyes were developed to label mitochondria, including JC-1, TMRE/TMRM, and the MitoTracker dyes. Many of these probes are still used today, but they all suffer from a variety of shortcoming, including electron transport chain inhibition and quenching effects (Perry et al., 2011).

1.2.4.2 Fluorescent proteins

The discovery and directed evolution of fluorescent proteins further expanded the role of fluorescence microscopy in mitochondrial research. In 1962, Osamu Shimomura identified green fluorescent protein (GFP) from dried samples of Aequorea victoria light organs (Shimomura, 2009). Thirty years later, Douglas Prasher was the first to clone and sequence GFP cDNA (Prasher et al., 1992). Realizing the tremendous potential of GFP as a molecular reporter, Martin Chalfie collaborated with Prasher, and demonstrated that GFP displayed bright and stable fluorescence when expressed in E. coli and C. elegans (Chalfie et al., 1994). Importantly, GFP did not appear to be toxic to cells and thus could be used to quantitatively monitor gene expression or protein localization.
However, wildtype GFP had excitation peaks at 390nm and 470nm, with peak emission at 509 nm (Chalfie et al., 1994). Consequently, imaging GFP required high energy, cytotoxic light and was difficult to multiplex with other fluorescent signals. To remedy this problem, Roger Tsien and colleagues used site directed mutagenesis to substitute key residues in wildtype GFP. In doing so, he engineered numerous stable, spectral variants of GFP (Heim & Tsien, 1996) These new fluorescent proteins could then be fused to cDNA encoding a mitochondria matrix targeting sequence and expressed in mammalian systems to identify mitochondria (Rizzuto et al., 1995; Rizzuto et al., 1996).

Since the mid-1990s, dozens of fluorescent proteins with excitation maxima across the visible spectrum have been discovered or engineered. These proteins vary in their brightness, stability, folding time, acid sensitivity, stokes shift, and fluorescence lifetime. Consequently, great care must be taken to ensure fluorescent proteins are both appropriate for a given biological question and practical for use with a specific microscope. Additionally, fluorescent protein biosensors have been developed which can be used to interrogate mitochondrial functions such as calcium content (Suzuki et al., 2016), ATP production (Imamura et al., 2009; Berg et al., 2009), and ROS generation (Ermakova et al., 2014).

Many of the major advances in understanding dynamic mitochondrial behavior have emerged since the development of these fluorescent proteins. However, a shortcoming of fluorescence microscopy is that it only allows the visualization of specifically tagged structures. Consequently, it can lead to an underestimation of the massive complexity and interconnectivity of living cells. Two of the major themes in modern mitochondrial research are the importance of interactions between (1) mitochondria and other
organelles, and (2) mitochondria and the cytoskeleton. In the next section, I will briefly review several recent developments in our understanding of mitochondria/cytoskeleton interactions.
1.3 Mitochondria-Cytoskeleton interactions

Mitochondria are complex organelles that coordinate numerous cellular functions, including reactive oxygen species signaling, Ca2+-buffering, lipid synthesis, and perhaps most notably, the generation of ATP by oxidative phosphorylation (Friedman & Nunnari, 2014). In contrast to the archetypal bean-shaped structure often seen in biology textbooks, mitochondria are in fact morphologically diverse and can form dynamic, highly complex networks that can dynamically rearrange their architecture in order to support cellular needs. This plasticity allows cells to quickly adapt to local changes in the extracellular milieu. Mitochondria network architecture is determined by multiple factors, including (1) the regulation of dynamin-like proteins on individual mitochondria (van der Bliek et al., 2014), (2) interactions between mitochondria and neighboring organelles (Murley & Nunnari, 2016), and (3) direct associations between mitochondria and the cytoskeleton (Figure 1). In this section, I will examine recent developments in our understanding of how the microtubule and actin cytoskeletons regulate mitochondria structure and function.

Fig. 1.2 A. Schematic of mitochondria (red), microtubules (blue), and f-actin (green) distribution in an undifferentiated cell. B. Mitochondria associate with microtubules (blue, bottom) and with actin (green, top) via motor/adaptor complexes. Dynemin/dynactin associate with mitochondria via TRAK and Miro to drive retrograde mitochondrial motility. In contrast, Kinesin-1 coordinates anterograde motility towards the cell periphery. Myo19 can associate with the mitochondria outer membrane either directly or through Miro. Syntaphilin anchors mitochondria to microtubules. C) Spinning disk confocal image of a HeLa cell expressing a mitochondria matrix marker (Mito-DsRed2) as well as markers for microtubules (top, SIR-Tubulin) and filamentous actin (bottom, LifeAct-GFP).
1.3.1 Dynamic interactions of mitochondria with microtubules

Mitochondria account for a small fraction (>10%) of the overall cell volume of interphase cells, but are highly dynamic, exploring ~80% of the cell volume within 15 min in some cell types (Valm et al., 2017). In mammalian cells, these dynamics are primarily driven by molecular motors actively translocating mitochondria along the microtubule cytoskeleton. In undifferentiated cells, microtubules are radially organized with their minus ends clustered at or near the microtubule-organizing center, and their more dynamic plus ends radiating outward toward the cell periphery. Cytoplasmic dynein and its activator dynactin drive the motility of mitochondria inward, toward the microtubule minus-end, while kinesin-1 is the major plus-end directed motor on mitochondria (Schwarz, 2014). The importance of mitochondrial-microtubule interactions has been highlighted by genetic and cellular studies, reviewed below, and also explored in theoretical work highlighting the role of the microtubule cytoskeleton in promoting structural heterogeneity and adaptive responsiveness of the mitochondrial network (Sukhorukov & Meyer-Hermann, 2015).

1.3.2 Miro and Milton

A genetic screen for mutants with impaired neuronal function in Drosophila led to the discovery of Milton (Stowers et al., 2002), a kinesin-binding protein that is critical for the regulation of mitochondrial motility. The mammalian homologs of Milton, TRAK1 and TRAK2, similarly mediate the motility of mitochondria through interactions with both kinesin and dynein/dynactin (van Spronsen et al., 2013). Milton and TRAKs 1 and 2 are scaffolding proteins that interact with mitochondria via the Ca^{2+}-binding GTPase Miro, which is anchored to the outer membrane of mitochondria via its tail domain (Guo et al.,
Increased cytosolic Ca²⁺ inhibits mitochondrial motility in a Miro-dependent manner (Wang & Schwarz, 2009; Macaskill et al., 2009).

Both genetic screens and targeted disruptions indicate that mitochondrial dynamics are critically important in differentiated cells with highly polarized morphologies such as neurons. In Drosophila, Miro mutants are lethal due to the impairment of mitochondrial trafficking in neurons (Guo et al., 2005), and knockout of Miro1 in mouse is sufficient to cause early postnatal death, while neuron-specific deletion of Miro1 results in both neurodevelopmental and neurodegenerative defects (Nguyen et al., 2014).

The neuronal phenotypes observed for Miro and Milton mutations indicate that the active translocation of mitochondria along microtubules is critically important for neuronal health. Mitochondrial motility is especially important during neuronal outgrowth, but this motility is dramatically reduced in mature neurons, with <10% of mitochondria motile along the axons of mature mammalian neurons both in vitro and in vivo (Lewis et al., 2016; Misgeld & Schwarz, 2017).

In axons, the mitochondrially-targeted protein syntaphilin contributes to the anchoring of stationary mitochondria to microtubules (Kang et al., 2008) leading to enrichment at presynaptic sites (Lewis et al., 2016). The protein Disrupted-In-Schizophrenia 1 (DISC1) interacts with syntaphilin, TRAK1, and Miro (Ogawa et al., 2014; Park et al., 2016) to modulate the anchoring activity of syntaphilin, and thus the Ca²⁺-dependent regulation of mitochondrial motility. Loss of this anchoring leads to defects in short-term facilitation, likely through defects in local mitochondrial-dependent Ca²⁺-buffering (Kang et al., 2008).
1.3.3 PINK1/Parkin

Turnover of aging and damaged mitochondria is a critical function in long-lived, post-mitotic cells such as neurons. Multiple studies have linked changes in mitochondrial motility to mitochondrial quality control pathways such as mitophagy. In one pathway, damaged mitochondria are immobilized along the axon through the PINK1- and Parkin-dependent degradation of Miro (Shlevkov et al., 2016), promoting localized mitophagy (Ashrafi et al., 2014). Parkinson’s disease-related mutations in PINK1, Parkin or LRRK2 slow the degradation of Miro, leading to the hypothesis that delayed arrest of damaged mitochondria along the axon also delays their degradation (Hsieh et al., 2016). However, it remains unclear if the localized degradation of stalled mitochondria via axonal mitophagy is the major mechanism for mitochondrial quality control in neurons, as in vivo studies indicate that most PINK1- and Parkin-dependent mitophagy occurs within the soma (Sung et al., 2016). A novel mechanism linking syntaphilin to the removal of stressed mitochondria has recently been described, in which the application of the respiratory complex III inhibitor Antimycin A1 to primary neurons induces the budding of syntaphilin-positive carrier vesicles from the ends of damaged mitochondria along axons. These vesicles are then transported in association with late endosomes toward the soma for lysosomal degradation (Lin et al., 2017). The increased prevalence of syntaphilin-positive vesicles in neurons from neurodegenerative disease models suggests that the formation of these vesicles may be a response to chronic stress, but the mechanism leading to their generation remains to be determined, as it is independent of both Parkin and Drp1 activity (Lin et al., 2017).
1.3.4 Linking mitochondrial function and position

While most studies to date have focused on the role of Ca\(^{2+}\) in regulating mitochondrial motility along microtubules, two other regulatory pathways have also been explored. Schwarz and colleagues looked at mechanisms connecting local energy state to mitochondrial motility, and found that the direct modification of Milton (TRAK1) by the enzyme O-GlcNAc transferase (OGT) in response to changes in the extracellular glucose concentration modulates mitochondrial motility in neurons (Pekkurnaz et al., 2014). This mechanism allows the cell to relocalize mitochondria in response to changes in the overall energy landscape. Recent work has also shown that ROS production can affect mitochondrial dynamics. Either exogenous or endogenously generated ROS inhibits mitochondrial motility in a pathway that is independent of changes in Ca\(^{2+}\), at least in mammalian cells, but is dependent on the MAP kinase p38a, potentially working through the Miro/TRAK adaptor complex (Debattisti et al., 2017). Of note, the mechanism is not limited to neurons, but also affects mitochondrial motility in cardiomyocytes. This observation is of potential interest, due to the importance of mitochondrial localization in cardiomyocytes (Miragoli et al., 2016). In failing heart cells, changes in the alignment of cellular microtubules lead to altered mitochondrial organization, which is correlated by defects in calcium release in response to mechanical stimuli. Thus, in both neuronal synapses and cardiomyocytes, the regulated interactions of mitochondria with microtubules are required to maintain normal calcium dynamics, highlighting the key role of mitochondria in maintaining cellular Ca\(^{2+}\) homeostasis, and the cell’s ability to dynamically relocalize mitochondria in response to changes in the external environment such as glucose or ROS levels.
A major role of mitochondria is to produce ATP, and recent work has provided new insights into the connection between mitochondrial motility along microtubules and energy gradients in cells. Schuler et al. (Schuler et al., 2017) used 3D fluorescence spinning disc confocal and lattice light sheet microscopy to monitor the intracellular response of the biosensor PercevalHR, which provides a ratiometric measure of ATP/ADP levels. In wild type MEFs, a gradient was observed with highest ATP:ADP ratios in the perinuclear region and a gradual decline in the ratio toward the cell periphery. A steeper gradient was observed in Miro-/- MEFs, where mitochondria were more strictly localized to the perinuclear region due to defective mitochondrial transport along microtubules. These differences were most apparent as deficits in energy-dependent processes such as membrane ruffling, leading edge protrusion, and focal adhesion dynamics at the cell periphery, leading to marked differences in cell migration. These observations are further confirmation of the long-held hypothesis that the active positioning of mitochondria near sites of energy need are required for normal cellular function.

1.3.5 Mitochondrial dynamics in mitosis

A dramatic change in the interaction of mitochondria with microtubules occurs as dividing cells enter mitosis. Prior to mitosis, many mitochondria are closely associated with the microtubule cytoskeleton, through the motor-dependent interactions described above. However, as cells enter prophase, the association of mitochondria with microtubules is significantly decreased, due to the CDK1- and Aurora A-dependent shedding of kinesin and dynein motors (Chung et al., 2016). This shedding has been proposed to allow for the passive segregation of mitochondria between daughter cells (Chung et al., 2016). Alternatively, mitochondrial distribution at the end of mitosis may be more active. As dividing cells initiate cytokinesis, mitochondria rebind to microtubules in a Miro- and
CENP-F dependent manner, and actually track with dynamic microtubule plus ends in a
dynamic mechanism that favors the equal inheritance of these organelles by the two
daughter cells (Kanfer et al., 2015). Also, as noted below, actin dynamics may facilitate
equitable organelle partitioning following cell division in mammalian cells.

1.3.6 Actin dynamics in mitochondrial motility and remodeling

Across the tree of life, the actin cytoskeleton plays important and diverse roles in regulating
mitochondrial network structure and function. In simple eukaryotes, such as budding
yeast, the actin cytoskeleton directs mitochondrial movement (Simon et al., 1995) and is
essential for the faithful inheritance of mitochondria upon cytokinesis (Boldogh et al., 2001)
In metazoans, long range mitochondrial movement is coordinated by microtubules, but the
actin cytoskeleton plays an important role in regulating mitochondrial distribution,
coordinating short range mitochondrial motility (Quintero et al., 2009), anchoring (Pathak
et al., 2010), and fission (Korobova et al., 2013; Manor et al., 2015; Li et al., 2015; Moore
et al., 2016).

1.3.7 Myosin XIX

Perhaps the most direct evidence for actin based mitochondrial motility came with the
discovery of the unconventional myosin motor, Myosin XIX (Myo19). A plus-end directed
motor with sequence similarity to MyoV, Myo19 was shown to specifically localize to the
mitochondrial outer membrane where it drives mitochondrial movement (Quintero et al.,
2009; Lu et al., 2014). Overexpression of fluorescent tagged Myo19 induced a nearly two-
fold increase in mitochondrial velocity, which was reversed upon depolymerization of actin
but not microtubules. Since its initial discovery, Myo19 has been implicated in a range of
functions, including targeting mitochondria to stress induced filopodia (Shneyer et al.,
2016; Shneyer et al, 2017) and coordinating mitochondrial inheritance upon cytokinesis (Rohn et al., 2014). Most recently, Myo19 localization to mitochondria was shown to be dependent on Miro proteins, suggesting that Miro may regulate both actin and microtubule based mitochondrial motility (Lopez-Domenech et al., 2018). In addition to Myo19, there is evidence that other myosin motors facilitate mitochondria dynamics. Work in Drosophila motor neurons indicates that Myosin V and VI can localize to axonal mitochondria in order to anchor mitochondria to actin filaments and oppose microtubule based motility (Pathak et al., 2010).

1.3.8 INF2/Spire1C mediated mitochondrial fission

In addition to its role in coordinating short-range mitochondrial motility, filamentous actin has also been implicated in the regulation of mitochondrial fission. Within the last few years, at least two distinct mechanisms of actin-mediated mitochondrial fission have been described (Figure 1.3). In nearly all cases, mitochondria fission occurs via the assembly and hydrolysis-mediated constriction of ring-shaped Drp1 oligomers on the mitochondrial outer membrane (Smirnova et al., 2001). Drp1 rings tighten and constrict mitochondria, facilitating the subsequent recruitment of dynamin-2 which ultimately coordinates membrane scission (Lee et al., 2016). However, because mitochondria are often thicker than the diameter of Drp1 rings, they must first undergo a pre-constriction step to decrease their cross-sectional diameter. This pre-constriction is achieved by wrapping and tightening endoplasmic reticulum tubules around mitochondria (Friedman et al., 2011).

Converging evidence suggests that ER bending around mitochondria is driven by the force of actin polymerization (Korobova et al., 2013; Manor et al., 2015). Work from the Higgs lab determined that an ER-anchored isoform of the formin INF2 is absolutely critical for
this process. Depletion of INF2, they found, resulted in increased mitochondrial length (Korobova et al., 2013). Subsequent work showed that INF2 cooperates with a mitochondria-localized Spire protein, Spire1C, in order to generate short actin filaments that span Mitochondria-ER contacts (Manor et al., 2015). The force of this actin assembly tightens the ER tubule around mitochondria. Myosin II likely contributes to actin-mediated mitochondrial constriction, as the motor localizes to fission sites and its depletion leads to mitochondrial elongation and loss of mitochondrial Drp1 (Korobova et al., 2014; Curchoe & Manor, 2017).

In addition to driving constriction, F-actin at Mito/ER contacts may also prime Drp1 to form functional oligomers on the mitochondrial outer membrane (DuBoff et al., 2012; Ji et al., 2015). In vitro GTPase assays show that Drp1 activity is greatly enhanced in the presence of actin filaments (Ji et al., 2015). More recent work has shown that INF2 driven actin assembly at mitochondria-ER contacts also triggers calcium uptake into the mitochondria matrix which then synchronizes outer and inner membrane fission (Chakrabarti et al., 2018). Taken together, these observations suggest that filamentous actin acts as an important regulator of inner and outer mitochondrial membrane fission.

Arp2/3 mediated mitochondrial fission.
Stress-induced mitochondrial fission through treatment with the mitochondrial poison CCCP was shown to promote rapid actin assembly around mitochondria (Li et al., 2015).
Strikingly, this actin polymerization is not dependent on INF2 and does not specifically occur at mito-ER contact sites. Instead, actin transiently assembles around whole mitochondria in an Arp2/3 dependent manner. These actin assemblies deform mitochondria and facilitate Drp1 recruitment and subsequent fission.

While CCCP treatment triggers rapid and reproducible mitochondrial fission, it nonetheless represents a non-physiological mechanism of mitochondria division. Importantly, there is strong evidence that Arp2/3 actin clouds may also play a role in homeostatic mitochondrial fission, even in the absence of a stressor. In wildtype MEFs, the actin binding proteins Arp3, cortactin, and coflin were shown to localize to mitochondria by immunofluorescence (Li et al., 2014; Pagliuso et al., 2016). Depletion of these proteins led to increased mitochondrial length and connectivity due to lower rates of fission (Li et al., 2014). Arp2/3-mediated mitochondrial fission may facilitate mitochondria network fragmentation at the G2/M transition as mitochondrial fission at prophase is linked to increased mitochondrial F-actin (Li et al., 2014).

Most recently, the dynamic, Arp2/3-dependent assembly of F-actin on mitochondrial subpopulations has been observed in multiple cell types, including HeLa cells, Cos7 cells, and human epidermal keratinocytes (Moore et al., 2016). Actin filaments transiently assemble in a ‘cloud’ around ~25% of cellular mitochondria, and then disassemble and subsequently reassemble around a neighboring subpopulation of mitochondria. Thus the actin cloud travels in a wave-like manner that propagates through the entire mitochondrial network over approximately 15 minutes. Upon actin assembly, mitochondria undergo rapid, Drp1-dependent fragmentation (Moore et al., 2016). This fragmentation is reversed following F-actin disassembly when fusion of neighboring mitochondria reinitiates.
Constitutive actin cycling has been proposed to function as a homeostatic mechanism by which the actin cytoskeleton regulates the mitochondrial network (Moore et al., 2016). Each round of transient fission prevents local hyperfusion of the mitochondrial network and the subsequent fusion following actin disassembly facilitates mixing of mitochondria contents.

1.3.9 Conclusions:

Dynamic interactions between mitochondria and the cytoskeleton are critically important to maintain mitochondria network structure and function. There has been a long-standing appreciation for the role of the cytoskeleton in mitochondrial motility. This motility is essential for intracellular shuttling to regions of high-energy demand or to locally buffer calcium. More recent work has highlighted the role of actin filaments and microtubules in the regulation of the mitochondrial fission/fusion balance, as well as mitochondrial quality control and turnover, and mitochondrial inheritance during cell division. Over the next few years, further advances in light microscopy techniques that allow for high-speed three-dimensional imaging of cells and tissues will continue to deepen our understanding of how these dynamic networks interact.
1.4 Mitochondrial quality control

The maintenance of mitochondrial health over the lifespan of an organism is a critically
important homeostatic function. Mitochondria are central to cellular health and
metabolism, as such, mitochondrial damage can induce acute cellular dysfunction through
either loss of mitochondrial function or through activation of inflammatory and cell death
pathways. To date, numerous mitochondrial quality control mechanisms have been
described, which attempt to mitigate mitochondrial damage. Here, I will briefly outline
these pathways:

1.4.1 Mitophagy

A portmanteau of “mitochondria” and “autophagy,” mitophagy refers to the wholesale
degradation of mitochondria by autophagosomal engulfment and subsequent lysosomal
acidification. Programmed mitophagy plays a key role in the normal development of
numerous cell types that do not require mitochondria, including erythrocytes, cornified
layers of the epidermis, and lens epithelium. Damage induced mitophagy, by comparison,
is a tightly coordinated quality control mechanism that numerous cells use to recognize
and remove depolarized or aged mitochondria.

The first description of mitophagy may stretch back over 100 years. Lewis & Lewis (1915)
showed that exposure of cells to acetic acid vapor induced the conversion of elongated,
rod-like mitochondria into small ring-shaped structures, or “degenerate mitochondria,”
which eventually disappeared into the surrounding cytoplasm. Nearly fifty years later,
electron microscopy allowed for the visualization of fragmented mitochondria
encapsulated by lysosomes (Clark, 1957; Ashford & Porter, 1962). Despite these early
observations, it would take several decades to piece together the precise mechanism of
mitophagy. In 2000, the identification of microtubule-associated protein 1 light chain 3 (LC3) as a bona-fide autophagosome membrane marker opened the door to the study of mammalian autophagy by fluorescence microscopy (Kabeya et al., 2000). Cultured cells could be transfected with cDNA encoding GFP-LC3 and Mito-RFP, and the dynamics of mitochondria and autophagosomes could be tracked before, during, and after mitochondrial damage.

In 2008, Youle and colleagues discovered that a gene mutated in familial Parkinson's disease – Parkin – is required for mitochondrial autophagy (Narendra et al., 2008). Using mammalian cell lines, the authors induced acute mitochondrial damage with the protonophore CCCP, and observed the recruitment of fluorescent parkin, and eventually LC3 to the damaged mitochondria. In a follow up paper, the authors implicated a second PD linked gene – PINK1 – in the initial recognition of mitochondrial damage (Narendra et al., 2010). Since these original observations, the PINK1/Parkin pathway has been extensively dissected and the mitophagy field has bourgeoned (Fig. 1.4).

Though specific details of the PINK1/Parkin pathway remain controversial, the general outline of the pathway can be summarized as follows:
PINK1 contains a short mitochondrial targeting sequence which drives import of the protein into the IMM. Once in the IMM, PINK1 is cleaved by resident proteases including MPP and PARL to form a shorter, 52 kDa form, which then translocates out of the mitochondria for degradation by the proteasome. Crucially, import of PINK1 is dependent on mitochondrial membrane potential ($\Delta \Psi_m$). Mitochondrial depolarization (through protein aggregation, redox stress, or acute intoxication by mitochondrial poisons) blocks PINK1 import and cleavage, resulting in stabilization of the longer, 64 kDa form of PINK1 on the OMM (Pickles et al., 2018).

Once stabilized on the OMM, PINK1 phosphorylates ubiquitin moieties present on the surface of mitochondrial proteins, which serves as a signal to recruit the cytosolic E3 ligase Parkin. Parkin, in turn, binds to phospho-ubiquitin on the OMM and ubiquitinates additional mitochondrial substrates, which are then phosphorylated by PINK1. This feed forward pathway results in the rapid formation of ubiquitin chains on the surface of damaged mitochondria, which either drive the proteasomal degradation of OMM proteins or recruit autophagic membranes for wholesale destruction of the mitochondrion (Lazarou et al., 2015).

Autophagy receptors, including optineurin (OPTN), TAX1BP1, and NDP52, bind to the ubiquitin chains via ubiquitin recognition motifs, and recruit the autophagic membrane protein LC3 via LC3 interacting region (LIR) motifs (Lazaoru et al., 2015; Wong & Holzbaur, 2015). The affinity of these autophagy receptors for either ubiquitin or LC3 is precisely tuned by TBK1 phosphorylation (Moore & Holzbaur, 2016; Heo et al, 2015).
Autophagosome membranes then assemble around the mitochondrion and form a sealed compartment that prevents the damaged organelle from triggering cell death or inflammatory pathways. The mitophagosome then fuses with numerous lysosomes and the subsumed mitochondrion is hydrolyzed and destroyed.

Disease-associated mutations in either Pink1 or Parkin interfere with this pathway, resulting in the accumulation of damaged or dysfunctional organelles. Further, Amyotrophic Lateral Sclerosis (ALS) linked mutations in OPTN and TBK1 have been shown to interfere with this pathway (Wong & Holzbaur, 2014; Moore & Holzbaur, 2016; Heo et al., 2015; Lazarou et al, 2015; Richter et al., 2016).

Critically, many of the damage paradigms used to activate mitophagy are often far more severe than the stressors that mitochondria might face \textit{in vivo}. Thus, mitophagy may be an extraordinary measure that is only used in instances of extreme, acute damage.

1.4.2 \textit{Stress induced mitochondrial hyperfusion}

Several groups have found cellular stress result in rapid morphological changes to mitochondrial networks. Among these changes is a phenomenon known as stress induced mitochondrial hyperfusion (SIMH). Whereas high levels of stress can trigger apoptosis and Drp1-mediated mitochondrial network fragmentation, low-to-moderate levels of stress instead drives mitochondrial fusion, resulting in the formation of a highly interconnected mitochondrial reticulum (Tondera et al., 2009). This SIMH requires the fusion proteins MFN1 and OPA1 and may drive increased ATP production. Importantly, this mitochondrial quality control mechanism is upregulated in response to physiologically relevant damage,
including UV irradiation (Tondera et al., 2009) and cigarette smoke (Ballweg et al., 2014; Bahl et al., 2016).

1.4.3 Mitochondria Derived Vesicles

In 2008, the McBride group identified mitochondria derived vesicles (MDV), small ~100-120nm structures that bud off of the surface of mitochondria in a Drp1-independent manner (Neuspiel et al., 2008). MDVs were originally found to play an important role in targeting an OMM protein called MAPL to a population of peroxisomes. However, follow up investigations from the same group showed that MDVs could actually target diverse mitochondrial cargoes directly to lysosomes for degradation. Of note, this pathway did not require the essential macroautophagy genes ATG5 or LC3, suggesting that MDVs represent a wholly distinct mechanism of piecemeal mitochondrial turnover (Soubannier et al., 2012).

MDV biogenesis was shown to upregulate in response to sublethal oxidative stress, including incubation of cells with xanthine oxidase or Antimycin A (Soubannier et al., 2012). Further, in vitro assays showed that MDVs frequently contained oxidized proteins. Thus, MDVs likely function as a mechanism to selectively eliminate oxidized mitochondrial components without completely eliminating the organelle (Sugiura et al., 2014).

1.4.4 Mitochondrial proteases

A major consequence of oxidative phosphorylation is electron leak and the generation of free radicals. Mitochondria contain an enzyme called superoxide dismutase, which catalyzes the conversion of superoxide into hydrogen peroxide, which is then further
decomposed by catalase into water and oxygen. Despite this antioxidant defense mechanism, mitochondrial proteins are highly susceptible to oxidative stress. Consequently, mitochondria contain several resident proteases which coordinate the turnover of oxidized proteins. Additionally, these proteases are involved in degrading proteins that have not been folded properly by mitochondrial chaperones.

1.4.5 Mitochondria off-loading by TNTs

In addition to the “vertical” mitochondria network inheritance during cell division, individual mitochondria can also pass “horizontally” between interphase cells via intercellular membrane conduits known as tunneling nanotubes (TNTs). TNTs are thin, cytoplasmic extensions that link together multiple cells. These membranous connections measure between 20 and 500 nm in diameter and can stretch for over 100 microns in length (Wang & Gerdes, 2015). TNTs can transport numerous cargoes between cells and have been identified in a broad number of cell types including neurons (Victoria et al., 2016), cardiomyocytes (Koyanagi et al., 2005), mesenchymal stem cells, and human cancer cells (Lou et al., 2012). Mitochondria transfer by TNTs has been shown to rescue apoptotic PC12 cells (Wang & Gerdes, 2015) and ameliorate mtDNA associated defects in osteosarcoma cells (Chuang et al., 2017).

Recent work has shown that TNTs are predominantly actin-based structures but can also be supported by microtubule networks. However, high resolution imaging of TNT organization has been challenging due to their small size and lack of association with the substratum. To date, several cargoes have been identified in TNTs, including viral particles, endoplasmic reticulum, and mitochondria. The mechanism by which
mitochondria are targeted to TNTs, transported directionally through TNTs, and integrated into the recipient cell’s mitochondrial network are not well understood.

1.5 An obligate endosymbiont

Approximately 2-3 billion years ago, a highly unlikely event occurred whereby a free-living prokaryote was engulfed by a second unicellular organism, and, instead of being destroyed, successfully entered into an endosymbiotic relationship (Sagan, 1967). The mutually beneficial relationship provided the host cell with an enormous boost in energy production and afforded the subsumed symbiont a safe, metabolite-rich environment in which to grow and divide. The marked increase in cellular energy generation enabled the host cell to evolve a complex genome and develop traits that were previously unattainable in simple prokaryotes. Over time, the endosymbiont surrendered many of the functions required for free-living, and its genome decreased in complexity. Concurrently, the host cell developed complex protein import machinery and began to populate the proto-mitochondrion with an increasing number of nuclear encoded proteins. Today, approximately 98.8% of mitochondrial proteins are encoded in the nucleus (data from Human Protein Atlas).

1.5.1 mtDNA

Despite this overwhelming reliance on nuclear DNA, mitochondria still retain a vestige of their origin as free-living bacteria; a 16.5Kb genetic code. The metazoan mitochondrial genome encodes 37 total genes, including 13 for proteins that form core electron transport
chain complex subunits, 2 for rRNAs* which form resident mitochondrial ribosomes, and 22 for tRNAs which are required for the translation of the mtDNA proteome (Wallace & Chalkia, 2013). Recent work suggests that human mtDNA may in fact be more complex than previously believed. In 2001, a Japanese group identified a short open reading frames (sORF) hidden in the 16S rRNA gene (Hashimoto et al., 2001). They found that the sORF encoded a short peptide called humanin and went on to demonstrate that the protein had potent cytoprotective effects (Hashimoto et al., 2001). More recently, a novel sORF was identified in the other mitochondrial rRNA gene, which was shown to encode a 16 amino acid peptide called MOTS-c (mitochondrial open reading frame of the 12S rRNA-c) (Lee et al., 2016). In response to metabolic stress, MOTS-c translocates to the nucleus where it binds to chromatin and alters transcription of genes regulating metabolism (Kim et al., 2018). The observation that mtDNA gene products can regulate nuclear gene expression underscores the stunning complexity of nuclear-mitochondria interactions and serves as a clear reminder of the mitochondrial endosymbiotic origin.

1.5.2 Heteroplasmy

Like nuclear DNA (nDNA), mtDNA cannot be generated de novo and must be replicated and inherited to pass to the next generation. However, the mitochondrial genome differs from the nuclear genome in several key areas. First, a normal diploid cell contains only one nuclear genome, but can have thousands of copies of mtDNA, each with its own unique complement of mutations. Second, mtDNA mutates at a significantly faster rate than nDNA and replicates more frequently than nDNA (Wallace, 1987). Finally, whereas the nuclear genome undergoes recombination during meiosis, the mitochondrial genome does not. As such, individual molecules of mtDNA will irreversibly accumulate genetic
mutations, a phenomenon known as Muller’s ratchet. The consequence of this is that many cells will have a mixed population of mitochondria; some healthy, others with possibly severe mutations. This mix of normal and mutated mtDNA within a single cell is known as heteroplasmic, and the ratio of heteroplasmic alleles can be an important determinant of cellular health. Every time a cell divides, a different percentage of mutant mitochondria can be inherited by each daughter, potentially resulting in the clonal expansion of mtDNA mutations and genetic drift between the resulting lineage Wallace and Chalkia, 2013).

1.5.3 Maternal mtDNA inheritance

Across all metazoans, mitochondria are uniparentally inherited via the maternal germ line. Consequently, the estimated $10^{17}$ mitochondria in the adult human body (Wallace and Chalkia, 2013) arise from a finite pool of mitochondria in the oocyte – estimated at ~92,500 organelles and containing ~2-3 pg of total mtDNA (Piko and Matsumoto, 1976). Due to this maternal inheritance, mtDNA mutations in the female germline can be passed down to offspring and are a frequent cause of mitochondrial diseases, including myoclonic epilepsy and ragged red fiber (MERRF), Leigh syndrome, or Leiber’s hereditary optic neuropathy (LHON). However, the penetrance of mitochondrial disease is determined by several factors, including the total burden of mtDNA mutations (heteroplasmic) and the type of cells that inherit defective mitochondria. Indeed, mitochondrial diseases typically affect cells with high energetic demand, such as neurons, skeletal muscle, and cardiomyocytes.
Maternal mtDNA inheritance is ensured by a highly coordinated mechanism of paternal mitochondrial elimination post-fertilization. In c. elegans, a two-step process occurs to ensure destruction of both mitochondria membranes and DNA. First, the paternal mitochondria inner membrane is destabilized, allowing a mitochondrial endonuclease G to translocate from the intermembrane space into the matrix where it degrades the paternal mtDNA (Zhou et al., 2016). Next, the rest of the organelle is targeted for degradation by ALLO-1/IKKE-1 mediated selective mitophagy (Sato et al, 2018). In mice, paternal mtDNA rarely persists past the 8-cell stage of development (Cummins et al., 1997). Intact mouse sperm was labeled with a green mitochondrial dye and microinjected into activated oocytes in order to induce embryonic development. By the 8-cell stage, over 85% of labeled sperm mitochondria were eliminated (Cummins et al., 1997).

In humans, paternal mtDNA was found in embryos of the eight-cell stage after in vitro fertilization (IVF), but persistence into further stages of fetal development is likely to result in spontaneous abortion. (St. John et al., 2000). In rare events, paternal mtDNA can be passed to offspring. Repeated interspecific crosses between inbred mouse strains has been associated with paternal mtDNA leak (Gyllensten et al., 1991). In humans, paternal mitochondrial inheritance has been identified and linked to mitochondrial dysfunction resulting in extreme exercise intolerance (Schwartz, 2002)

1.5.4 Somatic mtDNA inheritance

Throughout development and into adulthood, organisms must continue to generate new cells in order to grow and respond to injury. Each time a cell divides, it must not only coordinate the equal segregation of nuclear DNA, but also ensure the both symmetrical
and random inheritance of mtDNA. The random partitioning of mtDNA between daughter cells is critically important to prevent co-segregation of mtDNA mutants, potentially leading to the clonal expansion of those mutants, increased heteroplasmy, and genetic drift between daughter cell lineages.

To date, the mechanism of mitochondrial inheritance is not well understood. According to the passive inheritance model, mitochondrial inheritance occurs largely by a stochastic mechanism, in which passive, immotile mitochondria are randomly segregated based on their position relative to the division axis. Alternatively, active inheritance models suggest that mitochondria are preferentially targeted to daughter cells by either directed transport or selective tethering.

In asymmetrically dividing yeast, the actin cytoskeleton is involved in active mechanisms of mitochondrial inheritance. Upon bud formation, mitochondria are transited along actin cables via the class V myosin, myo2, and sorted into either mother or bud (Altmann et al., 2008). This myosin-based mitochondrial motility can be supplemented by an Arp2/3-dependent actin cloud that assembles on mitochondria and promotes their rapid movement over short distances (Boldogh et al., 2001).

In more complex eukaryotes, mitochondria primarily transit along microtubules. Due to this difference in track preference, it has been assumed that microtubules and microtubule-based motors would be responsible for mitochondrial inheritance. However, upon mitotic entry, the microtubule cytoskeleton undergoes a process of reorganization to form the mitotic spindle. Concurrent with this, mitochondria shed their microtubule-based motors so as not to interfere with spindle formation (Chung et al., 2016). Mitochondria do not re-
associate with microtubules until early anaphase, at which point they bind the microtubule plus-tip protein CENP-F via Miro and are targeted to daughter cells. This model assumes that free-floating mitochondria are evenly distributed throughout the cell, however, the work I present in chapter 6 indicates that this may not be the case.
CHAPTER 2: IMAGING THE DYNAMICS OF MITOPHAGY IN LIVE CELLS

2.1 Abstract

Investigating the precise spatiotemporal dynamics of mitophagy can provide insights into how mitochondrial quality control is regulated in different tissues and organisms. Here, we outline live-imaging assays to quantitatively assess mitophagy dynamics in real time. This protocol describes both chemical and optogenetic techniques to induce mitochondrial damage with high spatial and temporal control. Using these assays, mitochondria can be tracked from before they sustain damage up through their engulfment by autophagosomes and acidification by lysosomes.
Mitochondrial autophagy, termed mitophagy, is a tightly regulated degradative process by which damaged mitochondria are identified, sequestered, and then degraded. Within minutes of an initial precipitating injury leading to mitochondrial depolarization, a feed-forward cascade is activated involving the ubiquitin kinase PINK1 and the E3 ubiquitin ligase Parkin (Nguyen et al. 2016). PINK1 levels are normally low on healthy mitochondria, but mitochondrial depolarization leads to the accumulation of this kinase on the outer mitochondrial membrane and activation via autophosphorylation. The PINK1-dependent phosphorylation of ubiquitin, present at basal levels on the outer mitochondrial membrane, leads to recruitment of Parkin, which binds phospho-ubiquitin (p65-Ub) with high affinity (Nguyen et al. 2016). Once recruited, Parkin rapidly ubiquitinates mitochondrial proteins. The resulting high local concentration of ubiquitinated proteins actively recruits ubiquitin-binding adaptor proteins, including optineurin (OPTN), NDP-52, TAX1BP1, and NBR1 (Wong and Holzbaur 2014; Lazarou et al. 2015; Moore and Holzbaur 2016). The recruitment of these adaptor proteins has been visualized by live cell imaging (Wong and Holzbaur 2014; Moore and Holzbaur 2016), and occurs within 45 min of damage to mitochondria by either uncouplers such as CCCP or local generation of ROS by optogenetic activation of Mito-KillerRed (Wang et al. 2012; Wong and Holzbaur 2014). Multi-color live imaging has established the relative timing of receptor recruitment in HeLa cells, as well as the parallel recruitment of the kinase TBK1 (Moore and Holzbaur 2016).

Activation of the local autophagy machinery leads to the generation of LC3-positive isolation membranes. The mitophagy receptors OPTN and NDP52 have LC3-binding
LIR motifs that mediate the association of these isolation membranes with damaged mitochondria. The engulfment of damaged mitochondria can be readily visualized by live imaging using a spinning disk confocal microscope (Wong and Holzbaur 2014; Moore and Holzbaur 2016). Of note, mutations in either OPTN or TBK1 are causative for rare forms of familial ALS as well as glaucoma. Live imaging reveals that ALS-linked mutations in OPTN or TBK1 significantly disrupt LC3 recruitment, and thus the effective engulfment of damaged mitochondria (Moore and Holzbaur 2016; Wong and Holzbaur 2014).

Once a damaged mitochondrion is engulfed, the fully formed autophagosome will fuse with lysosomes over a period of several hours, facilitating the degradation of the organelle. This fusion can be visualized using a tandem mCherry-EGFP-LC3 construct (Pankiv et al. 2007). Initially, this construct will be visible in both the mCherry and GFP channels, but as the autophagosome becomes acidified by lysosomal fusion, the GFP fluorescence will be preferentially quenched, and only the mCherry fluorescence will remain. The acidification of the autophagosome can also be monitored by a pH-sensitive, cell permeant reporter such as Lysotracker.

Here we detail three protocols using multi-color live-cell imaging to monitor the dynamics of mitophagy. We describe protocols to induce mitophagy either by chemical or optogenetic strategies. Also, we outline use of pH sensitive reporters to monitor autophagosome formation and acidification by lysosomes.
2.3 Materials

2.3.1 HeLa Cell culture

1. HeLa Cells (CCL-2) from the American Type Culture Collection (ATCC) (see note 1)
2. 1x Dulbecco’s phosphate buffered saline (dPBS)
3. 0.25% Trypsin
4. Dulbecco’s modified Eagle medium (DMEM) with 4.5g/L glucose & L-glutamine (Corning, 10-017-CM)
5. Fetal bovine serum (FBS)
6. Glutamax
7. Growth medium: DMEM + 10% (v/v) FBS + 2mM Glutamax
8. Imaging medium: DMEM + 10% (v/v) FBS + 2mM Glutamax + 25mM HEPES
9. Plastic tissue culture dishes, 100mm
10. Glass-bottom Microwell Dishes, 35mm, No. 1.5 coverglass (e.g. MatTek, P35G-1.5-20-C)

2.3.2 Transfection

1. FuGENE 6 Transfection Reagent (Promega, E2692)
2. Opti-MEM Reduced Serum Media (Invitrogen, 31985070)
3. Mammalian expression constucts (see note 2)
   a. Mito-TagBFP (Addgene, 49151)
   b. EGFP-OPTN (Addgene, 27052)
   c. Mito-KillerRed (Evrogen, FP964)
   d. HaloTag-LC3B
e. mCherry-GFP-LC3B (Addgene, 22418)

f. Untagged Parkin (see note 3)

4. Far Red HaloTag Ligand (see note 4)

### 2.3.3 Live-imaging

1. Spinning-disk confocal microscope (e.g. UltraVIEW VoX 3D Live Cell imaging system (PerkinElmer) on a Nikon Eclipse Ti microscope)
2. Perfect focus system
3. EM-CCD camera (e.g. Hamamatsu, C9100-50)
4. 100X/1.49 NA Apochromat objective lens
5. 405nm, 488nm, 561nm, 640nm laser lines
6. Photobleaching module (e.g. UltraVIEW PhotoKinesis accessory (PerkinElmer))
7. Volocity acquisition software (PerkinElmer)

### 2.3.4 Chemicals and Dyes

1. CCCP (Sigma, C2759)
2. TMRE (ThermoFisher, T669)
3. LysoTracker Deep Red (ThermoFisher, L12492)

### 2.3.5 Image Analysis Programs

1. FIJI (Schindelin et al. 2012)

### 2.4 Methods
2.4.1 HeLa cell passaging protocol

1. Warm complete DMEM, 0.25% Trypsin, and 1X dPBS in 37°C water bath for at least 15 minutes.
2. Remove 10 cm dish of HeLa cells from incubator and place in biosafety cabinet.
3. Aspirate media and wash with 10mL of warm dPBS.
4. Aspirate dPBS and replace with 1 mL of 0.25% trypsin. Place dish in 37°C incubator for 5-7 minutes.
5. While cells are trypsinizing, add 12mL of warm, complete DMEM to a new 10cm dish and 2mL complete medium to a 35mm glass bottom dish.
6. Remove dish of trypsinized HeLa cells from the incubator and transfer to biosafety cabinet. Add 4mL of warm, complete DMEM to the HeLa cells and pipette up and down to dislodge remaining adherent cells. Transfer 5 mL of cell suspension to 15mL conical tube and centrifuge for 2 min at 500g.
7. Aspirate supernatant and resuspend cell pellet in 1mL complete DMEM.
8. Count cells using a hemocytometer. Seed 2,000,000 cells on the 10cm dish. These will be ready to split again in 48 hours. Seed 300,000 cells on the 35mm glass bottom dishes. These cells can be transfected in 24h.

2.4.2 Chemical Depolarization Mitophagy Protocol

2.4.2.1 Transfection

1. Before transfection, inspect the plate to ensure cells are ~40-60% confluent.
2. In a biosafety cabinet, add 100 µL OptiMem to a 1.5mL microcentrifuge tube.
3. Add 500ng of each plasmid you plan to transfec. Tap to mix. (see note 5)
a. TagBFP-Mito
b. EGFP-Optineurin
c. HaloTag-LC3B
d. Untagged-Parkin

4. Add 6µL FuGene6 Transfection Solution for a 3:1 ratio to plasmid DNA. Tap to mix.

5. Incubate at room temperature for 10 minutes.

6. Add transfection mix to your cells in a dropwise manner and return plates to 37C

2.4.2.2 Imaging

7. Approximately 23 hours after TFX, remove cells from incubator and gently replace medium with 2mL pre-warmed imaging medium containing 30nM TMRE and 100nM far-red Halo Tag ligand. (see note 6)

8. Allow cells to equilibrate for 45 minutes at 37C. (see note 7)

9. Remove 500µL of medium from the plate and transfer to a microcentrifuge tube.
   Add 2µL of 20mM CCCP and incubate at 37C.

10. Transfer cells to 37C heated microscope stage.

11. Manually adjust the objective height with the focus knob until reaching the desired focal plane.

12. Identify a cell expressing the constructs of interest with clear TMRE labeling of mitochondria. (see note 8)

13. Add back the 500µL of medium containing CCCP for a final concentration of 20μM. This concentration of CCCP should robustly depolarize mitochondria as indicated by loss of TMRE intensity. (see note 9)
14. Acquire a 4-color time-lapse movie of the cell at 1 frame per minute for 90 minutes.

2.4.3  

**Optogenetic Mitophagy protocol**

2.4.3.1  

**Transfection**

1. Before transfection, inspect the plate to ensure cells are ~40-60% confluent.
2. In a biosafety cabinet, add 100 µL OptiMem to a 1.5mL microcentrifuge tube.
3. Add 500ng of each plasmid you plan to transfect. Tap to mix.
   a. TagBFP-Mito
   b. EGFP-Optineurin
   c. Mito-KillerRed (Mito-KR)
   d. HaloTag-LC3B
   e. Untagged Parkin
4. Add 7.5 µL FuGene6 Transfection Solution for a 3:1 ratio to plasmid DNA. Tap to mix.
5. Incubate at room temperature for 10 minutes.
6. Add transfection mix to your cells in a dropwise manner and return plates to 37C incubator.

2.4.3.2  

**Imaging**

7. Approximately 23 hours after TFX, remove cells from incubator and gently replace medium with 2mL pre-warmed imaging medium containing 100nM far-red HaloTag ligand. Allow cells to equilibrate for 45 minutes at 37C.
8. Transfer cells to 37C heated microscope stage.
9. Manually adjust the objective height with the focus knob until reaching the desired focal plane.

10. Identify a cell expressing the constructs of interest. Take care to minimize green light exposure when scanning the plate as Mito-KR is a potent photosensitizer.

11. Using a 561nm laser, bleach a \( \sim 225\mu\text{m}^2 \) region of the mitochondrial network. Typically, we use 100% laser power and bleach for no longer than 1 minute.

12. Acquire a 4-color time-lapse movie of the cell at 1 frame per minute for 90 minutes. Within 45 minutes we generally begin to observe LC3 recruitment to mitochondria within the bleach window (Figure 2.1).
2.4.4 Lysotracker staining of acidified autophagosomes

2.4.4.1 Transfection

1. Before transfection, inspect the plate to ensure cells are ~40-60% confluent.
2. In a biosafety cabinet, add 100 µL OptiMem to a 1.5mL microcentrifuge tube
3. Add 500ng of each plasmid you plan to transfect. Tap to mix.
   a. TagBFP-Mito
   b. EGFP-mCherry-LC3B
   c. Untagged Parkin

Fig. 2.1. 45 minutes after bleach of Mito-KR, OPTN and LC3 can be visualized around the stable Mito marker (left column). In the same cell, unbleached mitochondria show robust Mito-KR fluorescence and no OPTN or LC3 recruitment (right column).
4. Add 4.5 µL FuGene6 Transfection Solution for a 3:1 ratio to plasmid DNA. Tap to mix.
5. Incubate at room temperature for 10 minutes.
6. Add transfection mix to your cells in a dropwise manner and return plates to 37°C incubator.

2.4.4.2 Imaging

7. Approximately 22 hours after TFX, replace medium with pre-warmed complete DMEM containing 75 nM LysoTrackerDeepRed. Allow cells to incubate for 1 hour.
8. Replace staining solution with 2mL pre-warmed imaging medium. Allow cells to equilibrate for 30 minutes at 37°C.
9. Remove 500µL of medium from the plate and transfer to a microcentrifuge tube. Add 2µL of 20mM CCCP and incubate at 37°C.
10. Transfer cells to 37°C heated microscope stage.
11. Manually adjust the objective height with the focus knob until reaching the desired focal plane.
12. Identify a cell expressing the constructs of interest.
13. Add back the 500µL of medium containing CCCP for a final concentration of 20µM.
14. Acquire a 4 color time-lapse movie of the cell at 1 frame per 2 minutes for 3 hours. (see note 10)

2.4.5 Assessing the Autophagic Engulfment of Depolarized Mitochondria with FIJI

1. Open time-lapse movie of CCCP treated cell in FIJI.
2. At each time point, use the multi-point tool to count the total number of 1) mitochondria, 2) OPTN-positive mitochondria, and 3) LC3-positive mitochondria. (see note 11)

3. For each time point, divide the number of OPTN-positive mitochondria by the total number of mitochondria. Repeat for LC3-positive mitochondria.

4. Plot the ratio of OPTN-positive mitochondria and LC3-positive mitochondria over time.

2.4.6 Assessing the Autophagic Engulfment of MitoKR Bleached Mitochondria in FIJI

1. Open time-lapse movie in FIJI.

2. Identify a bleached mitochondrion labeled with Mito-TagBFP but not Mito-KR.

3. Using the line tool, draw a 2 µm line scan through the organelle. Open the ROI manager (Analyze>tools>ROI manager) and select “Add” to save the position on the line scan.

4. Measure the mean intensity (Analyze>measure) along the line scan for each channel.

5. Move to the next time point and adjust the location of the line scan to correct for movement of the mitochondria. Add the position of the new line scan to the ROI manager and measure the intensity of all channels. Repeat for all time points.

6. Next, repeat this protocol for a Mito-KR labeled mitochondrion that was not bleached.

7. Plot the fluorescence intensity over time for OPTN and LC3. Compare between bleach and unbleached conditions.
2.4.7 Assessing the Lysosomal Acidification of Mitochondria

1. Open time-lapse movie in FIJI

2. Identify a mitochondrion and use the line tool to draw a 2µm line scan through the organelle. Add the line scan to the ROI manager as previously described.

3. For each time point, measure the mean intensity for the EGFP, mCherry, and LysoTracker Deep Red channels.

4. Plot the ratio of EGFP to mCherry intensity. Decreases in this ratio indicate quenching of EGFP fluorescence due to lysosomal acidification (Figure 2.2).

5. Simultaneously, plot the intensity of LysoTracker for each time point. Increased LysoTracker intensity indicates colocalization of lysosomes with mitochondria-positive autophagosomes.
2.5 Notes

1. We have used this protocol to visualize mitophagy in HeLa cells and Cos7 cells. We expect this protocol can be adapted to numerous other cell lines.

2. All of these constructs use CMV promoters, which allows for robust, constitutive expression in mammalian cells.
3. HeLa cells express low levels of Parkin, thus we express exogenous Parkin to facilitate mitophagy. It is also possible to generate stable, Parkin expressing cell lines using retroviral infection of untagged Parkin (Addgene, 89299) followed by limiting dilution to isolate monoclonal colonies.

4. HaloTag fusion proteins can be labeled by various cell permeant fluorescent ligands. For this assay, we used a far-red ligand (JF646 (Grimm et al. 2015)) to avoid spectral overlap with our other fluorescent probes.

5. We generally observe very high levels of co-transfection. If you observe low levels of co-transfection, be sure to thoroughly mix the plasmid solution before addition of FuGene 6.

6. At these concentrations, TMRE and JF646 can be used without a subsequent washing step. HaloTag-LC3 can also be labeled with a red ligand, but TMRE must be omitted from the assay.

7. HEPES buffered imaging medium allows for long term (~4h) imaging at atmospheric CO2 levels. After addition of imaging medium, cells should not be returned to a 5% CO2 incubator.

8. To avoid artifacts associated with overexpression in transient transfection assays, select cells with lower fluorescence intensity. We typically scan the plate to determine the range of expression levels for each channel. Then, we select cells in the lowest quartile of fluorescence intensities, provided the signal to noise ratio of each channel is sufficient to visualize structures of interest. If necessary, expression time after transfection can be reduced to as little as 16 hours. Alternatively, monoclonal stable cell line can be generated to provide more consistent expression levels for analysis.
9. TMRE import into mitochondria is dependent on membrane potential. When TMRE is used in non-quenching mode (we find that 30 nM works well), depolarization by CCCP should result in decreased TMRE intensity in mitochondria (Perry et al. 2011). Other chemicals, such as a combination of antimycin with oligomycin can also robustly depolarize mitochondria and trigger mitophagy.

10. In HeLa cells, autophagic engulfment of mitochondria occurs approximately 45 minutes to 1 hour after damage. In order to visualize acidification of autophagosomes it is necessary to image over a longer time window. To reduce the phototoxic effects of prolonged imaging, we recommend imaging at a slower frame rate.

11. We classify mitochondria as OPTN- or LC3-positive only when we can observe a clear ring of fluorescence around the rounded, fragmented mitochondrion.
CHAPTER 3: DYNAMIC RECRUITMENT AND ACTIVATION OF ALS-ASSOCIATED TBK1 WITH ITS TARGET OPTINEURIN ARE REQUIRED FOR EFFICIENT MITOPHAGY

This chapter is adapted from:


Supplementary movies can be accessed at:
http://www.pnas.org/content/113/24/E3349/tab-figures-data
3.1 Abstract

Mitochondria play an essential role in maintaining cellular homeostasis. The removal of damaged or depolarized mitochondria occurs via mitophagy, in which damaged mitochondria are targeted for degradation via ubiquitination induced by PINK1 and Parkin. Mitophagy receptors, including Optineurin (OPTN), NDP52, and TAX1BP1, are recruited to mitochondria via ubiquitin binding and mediate autophagic engulfment through their association with LC3. Here, we use live cell imaging to demonstrate that OPTN, NDP52, and TAX1BP1 are recruited to mitochondria with similar kinetics following either mitochondrial depolarization or localized generation of reactive oxygen species (ROS), leading to sequestration by the autophagosome within ~45 minutes after insult. Despite this co-recruitment, we find that depletion of OPTN, but not NDP52, significantly slows the efficiency of sequestration. OPTN is phosphorylated by the kinase TBK1 at Serine 177; we find that TBK1 is co-recruited with OPTN to depolarized mitochondria. Inhibition or depletion of TBK1, or expression of Amyotrophic Lateral Sclerosis (ALS) associated OPTN or TBK1 mutants blocks efficient autophagosome formation. Together, these results indicate that while there is some functional redundancy among mitophagy receptors, efficient sequestration of damaged mitochondria in response to mitochondrial stress requires both TBK1 and OPTN. Notably, ALS-linked mutations in OPTN and TBK1 can interfere with mitophagy, suggesting that inefficient turnover of damaged mitochondria may represent a key pathophysiological mechanism contributing to neurodegenerative disease.
3.2 Introduction

Mitochondria form interconnected networks that continuously remodel in response to shifting cellular needs (McBride et al., 2006). These dynamic networks serve as hubs for diverse cellular functions, including aerobic metabolism, calcium homeostasis (Pizzo et al., 2012), and redox signaling (Chandel, 2014). Several key mitochondrial functions rely on the potential across the mitochondrial inner membrane. Loss of membrane potential is associated with mitochondrial fragmentation, impaired trafficking (Wang et al., 2011) and can potentially activate cell death pathways (Summers et al., 2014). To safeguard against these deleterious outcomes, eukaryotic cells have developed quality control mechanisms to monitor the membrane potential of resident mitochondria and selectively eliminate depolarized organelles through mitophagy.

In mitophagy, damaged mitochondria are recognized and then sequestered by a double-membrane autophagosome, leading to selective degradation. Regulation of this process involves the ubiquitin kinase PINK1 (Zheng and Hunter, 2014) and the E3-ubiquitin ligase Parkin (Narendra et al., 2008). Specifically, PINK1 accumulates on the surface of depolarized mitochondria, where it phosphorylates ubiquitin on local outer membrane proteins, resulting in the recruitment of Parkin (Narendra et al., 2008; Matsuda et al., 2010; Narendra et al., 2010; Kane et al., 2014; Koyano et al., 2014). Parkin, in turn, modifies additional mitochondrial outer membrane proteins with ubiquitin linkages, which are subsequently phosphorylated by PINK1. The resultant phosphoubiquitin linkages further recruit and activate Parkin, initiating a feed-forward cascade resulting in the more extensive ubiquitination of mitochondria and the recruitment of the ALS-associated protein optineurin (OPTN) via its UBAN domain (Heo et al., 2015; Lazarou et al., 2015; Wong and Holzbaur, 2014). OPTN induces the formation of an LC3-positive autophagosome
that engulfs the damaged mitochondrion, effectively sequestering it from the cytosol and ensuring subsequent degradation. The recruitment of Parkin, OPTN, and LC3 to either depolarized or ROS-damaged mitochondria can occur in as little as 45 min after insult (Wong and Holzbaur, 2014), while complete degradation of engulfed mitochondrial fragments requires fusion of autophagosomes with lysosomes and can take up to 24 hours (Narendra et al., 2008).

OPTN is a member of a small class of proteins termed autophagy receptors, which, to date, includes at least four other members: p62, NDP52, TAX1BP1, and Nbr1 (Rogov et al., 2014). Like OPTN, these proteins bind both ubiquitin and LC3 to target ubiquitinated substrates to newly forming autophagosomes. However, the extent to which these other autophagy receptors participate in mitophagy and potentially cooperate with OPTN in the autophagic clearance of depolarized mitochondria is unclear (Heo et al., 2015; Lazarou et al., 2015; Wong and Holzbaur, 2014). Moreover, what role upstream regulators of these proteins, such as TANK Binding Kinase 1 (TBK1) (Wild et al., 2011), may play in this pathway remains unknown. While NDP52 and TAX1BP1 have recently been shown to be recruited to damaged mitochondria (Heo et al., 2015; Lazarou et al., 2015), the dynamics of this recruitment have not been investigated. Further, NDP52 has been proposed to function redundantly with OPTN during PINK1/Parkin mitophagy (Heo et al., 2015; Lazarou et al., 2015), but these studies focused primarily on downstream effects on mitochondrial clearance, rather than the efficiency of mitochondrial sequestration by autophagosomes.

Once sequestered within autophagosomes, damaged and potentially toxic mitochondria are functionally separated from the rest of the cell until they can be degraded by lysosomal
hydrolases. Thus, the rate at which acutely damaged mitochondria are engulfed by autophagosomes may potently affect cellular homeostasis, with delays leading to toxic cellular insult. Consistent with this possibility, mutations in OPTN cause neurodegeneration, including both ALS and glaucoma, while TBK1 mutations also result in ALS and are implicated in frontotemporal dementia (Cady et al., 2015; Freischmidt et al., 2015; Ito et al., 2011; Maruyama and Kawakami, 2013; Pottier et al., 2015; Weishaupt et al., 2013).

Here, we use live cell imaging to examine the dynamics of mitophagy induced by mitochondrial depolarization or localized ROS production. We find that OPTN is required for efficient mitophagy, although other receptors including NDP52 and TAX1BP1 are recruited to depolarized or damaged mitochondria with similar kinetics. OPTN recruitment occurs coincident with recruitment of the upstream serine/threonine kinase TBK1. TBK1-dependent phosphorylation of OPTN S177 is not required for OPTN recruitment to Parkin-positive mitochondrial fragments. However, phosphorylation of OPTN at S177 is required for the efficient formation of autophagosomes around depolarized mitochondria. Consistent with this, inhibition or depletion of TBK1, or expression of an ALS-associated TBK1 mutant, significantly impairs autophagic engulfment of depolarized mitochondria. Similarly, some, but not all, ALS-linked OPTN mutants disrupt the function of this receptor in mitophagy. Together, these data indicate that efficient sequestration of damaged mitochondria into LC3-positive autophagosomes requires both OPTN and TBK1.
3.3 Results

3.3.1 Dynamic recruitment of mitophagy receptors OPTN, NDP52, and TAX1BP1 to depolarized mitochondria.

We used multi-color live cell imaging to examine the dynamics of mitophagy following mitochondrial depolarization in HeLa cells. In control cells, mitochondria form dynamic, interconnected networks with low levels of mitochondrially-localized LC3 (Fig. 3.1A top panel). To induce mitochondrial depolarization, we treated HeLa cells with 20 µM CCCP, a protonophore that triggers rapid loss of mitochondrial membrane potential as indicated by the potentiometric dye TMRE (Fig. 3.S1A-C). As HeLa cells express low levels of endogenous Parkin (Pawlyk et al., 2003), CCCP treatment does not induce Parkin-dependent mitophagy in control cells, with less than 1% of mitochondria targeted to autophagosomes at 180 min post-CCCP treatment (Fig. 3.1B, 3.S1D). In contrast, engulfment of depolarized mitochondria can be clearly observed in HeLa cells expressing exogenous Parkin at 180 min post-CCCP, with 8% of mitochondria surrounded by identifiable LC3 rings (Fig. 3.1A,B). As previously shown (Wong & Holzbaur, 2014), knockdown of OPTN reduced mitophagy in Parkin-expressing cells, while concurrent expression of exogenous OPTN with Parkin resulted in a 4-fold increase in LC3-positive mitochondria at this time point (Fig. 3.1A,B), suggesting that cellular expression levels of OPTN may be rate-limiting.

In addition to OPTN, autophagy receptors NDP52 and TAX1BP1 possess the ubiquitin-binding and LC3 Interacting Region (LIR) motifs required to target ubiquitinated mitochondria to autophagosomes. NDP52 and TAX1BP1 have been shown to function along with OPTN in the autophagic clearance of invasive bacteria (Thurston et al., 2009;
Tumbarello et al., 2015; Wild et al., 2011), and more recently, these adaptors have been implicated in the long-term clearance of depolarized mitochondria (Lazarou et al., 2015; Heo et al., 2015). However, the relative dynamics with which OPTN, NDP52, and TAX1BP1 are recruited to depolarized mitochondria have not been examined. To investigate their recruitment using live cell assays, we transfected HeLa cells with untagged Parkin, Mito-DsRed2, GFP-LC3, and either Halo-OPTN, Halo-NDP52, or Halo-TAX1BP1.

Treating cells with 20 µM CCCP induced the recruitment of OPTN to fragmented mitochondria within 30 min. By 90 min post-CCCP, OPTN was stably enriched on over 50% of mitochondria (Fig. 3.1C,F). NDP52 and TAX1BP1 displayed analogous recruitment kinetics to OPTN, similarly translocating to depolarized mitochondria within 30 min of CCCP treatment. At 90 min post-depolarization, NDP52 localized around 50% of Mito-DsRed2 labeled mitochondrial fragments while TAX1BP1 formed visible rings around 59% of depolarized mitochondria (Fig. 3.1D-F). While these receptors displayed similar patterns of enrichment on depolarized mitochondria, they differed in their ability to facilitate LC3 recruitment and subsequent mitophagy. Among NDP52-positive mitochondria, 57% were clearly surrounded by GFP-LC3 rings (Fig. 3.1H), indicating efficient sequestration of depolarized mitochondria by the autophagic machinery. In contrast, only 35% of OPTN-positive mitochondria and 30% of TAX1BP1-positive mitochondria were engulfed by autophagosomes at 90 min post-CCCP (Fig. 3.1H). Of note, expression of exogenous OPTN, NDP52, or TAX1BP1 significantly enhanced mitophagy in Parkin-expressing cells as compared to cells expressing only endogenous levels of mitophagy receptors (Fig. 3.1G).
In parkin-expressing HeLa cells transfected with all three autophagy receptors together, we observe robust co-localization of OPTN, NDP52, and TAX1BP1 on mitochondria 90 min post-CCCP (Fig. 3.S2A). Recruitment of each of these receptors is dependent on Parkin E3-ligase activity, as expression of the Parkinson’s disease-associated T240R-Parkin mutant effectively blocked association of OPTN (Wong & Holzbaur, 2014), NDP52, or TAX1BP1 with mitochondria at 1h post-CCCP (Fig. 3.S2B,C). Although direct targeting of an engineered PINK1 construct to mitochondria was reported to be sufficient to recruit NDP52 (Lazarou et al., 2015), we find that endogenous PINK1, in the absence of parkin, is insufficient to recruit NDP52 (Fig. S2D).

3.3.2 OPTN, NDP52, and TAX1BP1 are recruited to damaged mitochondria with comparable kinetics.

We wondered whether the dynamics of OPTN, NDP52 and TAX1BP1 recruitment differed in response to focally induced mitochondrial damage via localized production of ROS. Cells were transfected with untagged Parkin, Mito-SNAP, GFP-LC3, and either Halo-OPTN, Halo- NDP52, or Halo-TAX1BP1. Cells were also transfected with Mito-KillerRed (MitoKR), a mitochondrially targeted photosensitizer that generates localized production of ROS upon 561 nm laser illumination (Yang and Yang, 2011). Prior to photobleaching, mitochondria double-labeled with MitoKR and Mito-SNAP were highly dynamic and indistinguishable in morphology from mitochondria in cells expressing Mito-DsRed2. We selected one region within each cell and photobleached the MitoKR signal with a 561 nm laser. After bleaching, the MitoKR signal was no longer visible, but mitochondria could still be identified by the photostable Mito-SNAP signal (Fig. 3.S3A).
At 90 min after MitoKR photobleaching, we observed robust recruitment of both OPTN and LC3 to bleached mitochondria, but not to mitochondria in unbleached regions of the cell (Fig. 3.2A). In parallel experiments with NDP52 and TAX1BP1, we observed similar recruitment of each receptor to photobleached mitochondria; LC3 was also recruited at this time point (3.2B-C).

Next, we investigated the kinetics of OPTN, NDP52, and TAX1BP1 recruitment in response to focal mitochondrial ROS production. Within 15-20 min of MitoKR activation, we observed Parkin recruitment to mitochondria (Fig. 3.S3B). Consistent with previous observations (Wong & Holzbaur, 2014), OPTN was recruited to bleached mitochondria ~25-30 min after activation of MitoKR (Fig. 3.3A, Movie 1). At this same time point, we observed the initial punctate recruitment of NDP52 to the surface of damaged mitochondria, and the formation of weak, but clearly discernible NDP52 rings around mitochondrial fragments (Fig. 3.3B, Movie 2). In experiments with TAX1BP1, initial recruitment was observed within 20-30 min, with defined TAX1BP1 rings formed around mitochondrial fragments by 30-40 min (Fig. 3.3C, Movie 3). In all experiments, LC3-positive rings could be observed forming around bleached mitochondria within 45 min. (Fig. 3.3A-C). To determine the rate of autophagosome formation around receptor-positive mitochondria we calculated the Pearson’s correlation coefficient between LC3 and either OPTN, NDP52, or TAX1BP1. In all cases, the Pearson’s coefficient between LC3 and autophagy receptor significantly increased within the bleach window but not within a distal unbleached region of equal size (Fig. 3.3D-F). Together, these results indicate that focal ROS production can initiate OPTN-, NDP52-, and TAX1BP1-dependent mitophagy within a subpopulation of cellular mitochondria.
While the precise kinetics show some variability from cell-to-cell or organelle-to-organelle, all three receptors tested in live cell assays were recruited subsequent to Parkin and prior to LC3. Notably, in our experiments with both MitoKR and CCCP, we observed a two-step recruitment process for OPTN and NDP52. In the 10-15 minutes immediately following Parkin translocation to damaged mitochondria, low levels of OPTN and NDP52 recruitment were observed, resulting in the formation of uniform, weakly fluorescent rings. When LC3 was subsequently recruited, we observed a robust increase in the autophagy receptor signal intensity (Fig. 3.4A-D). While the initial surge in OPTN/NDP52 intensity around mitochondria slightly diminished in the minutes after autophagosome formation, the subpopulation of mitochondria engulfed by LC3 at 90 min displayed significantly higher normalized autophagy receptor fluorescence as compared to mitochondria without LC3 (Fig. 3.4E-G). In contrast, the signal for mCherry-Parkin did not display a similar increase in intensity following mitochondrial engulfment by LC3-positive membranes (Fig. 3.4H).

We used fluorescence recovery after photobleaching (FRAP) to probe the stability of the mitochondrially bound receptors at 90 min post-CCCP, by photobleaching OPTN on mitochondria that either had or had not recruited LC3. (Fig. 3.4I-K). LC3-negative mitochondria (“-LC3”) with dim OPTN rings showed some recovery of fluorescence within 5 min of photobleaching. In contrast, LC3-positive mitochondria (“+LC3”) with bright OPTN rings at the time of photobleaching displayed no measurable OPTN recovery over the same time frame (Fig. 3.4L), suggesting that OPTN can cycle on and off of Parkin-positive mitochondria, but becomes stabilized as autophagosomes form.
3.3.3 Depletion of OPTN but not NDP52, blocks efficient mitophagy

There have been conflicting reports about the relative contribution of OPTN and NDP52 in mitophagy, and whether these receptors carry out redundant functions (Heo et al., 2015; Lazarou et al., 2015). To examine this question, we depleted Parkin-expressing cells of either NDP52 or OPTN, and investigated levels of mitophagy at 180 min post-CCCP. As previously observed (Wong & Holzbaur, 2014), knockdown of endogenous OPTN using siRNA leads to a significant defect in the autophagic engulfment of mitochondria (Fig. 3.5A,B). In contrast, depletion of NDP52 by siRNA (>95% depletion, Fig. 3.S4A) had no significant effect on autophagic engulfment of mitochondria at this time point (Fig. 3.5A,B).

Recent studies using stable OPTN-knockout HeLa cell lines reported no significant defects in mitochondrial clearance when assayed one day after mitochondrial depolarization (Heo et al., 2015; Lazarou et al., 2015). These observations are seemingly at odds with our observations concerning autophagic engulfment of mitochondria when assayed 180 min after mitochondrial depolarization. To investigate this question, we obtained OPTN and NDP52 knockout cells generated by Lazarou et al. (2015) and examined potential differences in mitophagy at time points more closely following mitochondrial depolarization. In the stable OPTN knockout cell line, we noted a modest but significant defect in mitophagy at 180 min post-CCCP (Fig. 3.S4B), consistent with our observations in cells with a transient depletion in OPTN (Fig. 3.5B). In contrast, no defect in mitophagy was observed in the NDP52 knockout cell line (Fig. 3.S4B), again consistent with our observations in cells in which NDP52 was transiently depleted by siRNA (Fig. 3.5B). Cells with stable knockout of both OPTN and NDP52 showed the most significant defects in mitochondrial engulfment by LC3 at this time point, paralleling the significant
defect in mitochondrial clearance at 24 h post-CCCP previously noted in this cell line (Lazarou et al., 2015). Thus, OPTN is required for effective mitophagy in the initial hours after mitochondrial damage, although other receptors, such as NDP52 likely compensate over longer timeframes (24 hours, Lazarou et al., 2015).

3.3.4 TBK1 is dynamically co-recruited to depolarized mitochondria with OPTN.

The serine/threonine kinase TBK1 has been implicated in the regulation of multiple receptors including p62, OPTN, and NDP52 (Matsumoto et al., 2015; Thurston et al., 2009). Additionally, immunocytochemistry studies indicate that TBK1 colocalizes with OPTN to intracellular pathogens as well as depolarized mitochondria (Matsumoto et al., 2015; Wild et al., 2011). Based on these observations, we chose to examine the dynamics of TBK1 recruitment in response to mitochondrial depolarization using live cell imaging. We transfected HeLa cells with SNAP-TBK1, GFP-OPTN, untagged Parkin, and Mito-DsRed2. Prior to CCCP treatment, both TBK1 and OPTN were distributed throughout the cytoplasm, occasionally localized to small, overlapping foci (Fig. 3.6A, top panel). Within 10 min of CCCP treatment, these TBK1/OPTN foci disappeared. Over the next 15 min, TBK1 and OPTN translocated onto mitochondria with similar kinetics (Fig. 3.6A,B, Movie 4). Once TBK1 was recruited to depolarized mitochondria, the kinase remained stably associated for over 90 min (Fig. 3.6C). The observed enrichment of TBK1 on depolarized mitochondria was eliminated by depletion of endogenous OPTN (Fig. 3.6D), suggesting that OPTN mediates TBK1 recruitment to mitochondria. Consistent with this hypothesis, overexpression of exogenous OPTN enhanced TBK1 association with depolarized, Parkin-positive mitochondria (Fig 3.6E). Finally, to determine whether TBK1 is co-
recruited with OPTN, we knocked down endogenous TBK1 and rescued with either wild
type TBK1 or an ALS-associated E696-TBK1 mutant that is deficient in OPTN binding
(Freischmidt et al., 2015). At 90 min post-CCCP, WT-TBK1 could be clearly visualized on
fragmented mitochondria (Fig. 3.6F, top panel). In contrast, E696K-TBK1 remained
cytosolic, failing to associate with depolarized mitochondria (Fig 3.6F). Thus, we propose
that OPTN shuttles TBK1 to depolarized mitochondria.

3.3.5 Activated TBK1 is necessary for efficient autophagic engulfment of depolarized
mitochondria.

A recent investigation found that TBK1 is activated through S172 phosphorylation after
mitochondrial depolarization (Heo et al., 2015). However, it is not known whether TBK1
activity is necessary for autophagic engulfment of depolarized mitochondria. To
investigate this, HeLa cells were transfected with untagged Parkin, Mito-DsRed2, GFP-
LC3, and either SNAP-TBK1 or Halo-OPTN. Approximately 24h later, we treated cells for
1h with either DMSO or 1 µM BX795, a small molecule inhibitor of TBK1 (Clark et al.,
2011). Prior to mitochondrial depolarization, cells treated with the TBK1 inhibitor were
indistinguishable from those that received vehicle control. At 90 min post-CCCP we
observed a clear enrichment of TBK1 on fragmented mitochondria (Fig. 3.S4A), indicating
that TBK1 recruitment to depolarized mitochondria is not dependent on its phosphorylation
state. Consistent with this, we also observed robust recruitment of phosphodeficient
S172A-TBK1 to depolarized mitochondria (Fig. 3.S4B). Next, we examined the effect of
TBK1 inhibition on OPTN recruitment and autophagosome formation. At 90 min post-
CCCP, 66% of mitochondria in cells treated with BX795 were surrounded by OPTN rings
(Fig. 3.7B,D), as compared to 55% of mitochondria in control cells (Fig. 3.7A,D). Despite this increased recruitment of OPTN, only 4.1% of OPTN-positive mitochondria were engulfed by LC3 in BX795 treated cells (Fig. 3.7F, 3.S5C). In contrast, 35% of OPTN-positive mitochondria in DMSO treated cells were engulfed by autophagosomes at 90 min post-CCCP (Fig. 3.7F). We observed a similar inhibition of mitophagy in BX795-treated cells depolarized with 10µM Antimycin together with 10µM Oligomycin (Fig. 3.S5D).

Depletion of TBK1 by siRNA (> 95% depletion; Fig. 3.S5E) resulted in a striking decrease in the percent of LC3-positive mitochondria following 90 min CCCP treatment, comparable to the effect observed in cells treated with BX795 (Fig. 3.6C,E,F). This mitophagy defect was rescued by the expression of wildtype TBK1, but not by a phosphodeficient S172A-TBK1 mutant (WT-TBK1, 20.11 ± 2.1%; S172A-TBK1 4.9 ± 0.5%, mean ± S.E.M.). Of note, knockdown of TBK1 resulted in a slight decrease in OPTN localization to depolarized mitochondria (Fig. 3.7D), suggesting that TBK1 may partially facilitate recruitment and/or retention of OPTN on mitochondria.

TBK1 inhibition or depletion also profoundly impaired the formation of LC3-positive autophagosomes around depolarized mitochondria in cells expressing endogenous levels of autophagy receptor (Fig. 3.7G). In fact, at 180 min post-CCCP, the inhibition of mitophagy observed due to depletion or inhibition of TBK1 was even more extensive than that observed upon OPTN knockdown (Fig. 3.7G).

Next, we investigated the effect of TBK1 inhibition on other autophagy receptors. We observed that inhibition of TBK1 by BX795 eliminated the mitophagy enhancement in cells overexpressing OPTN or TAX1BP1, but had only a minor effect on the upregulated
mitophagy observed in cells expressing NDP52 (Fig 3.7H,J). Both OPTN and NDP52 were still recruited to depolarized mitochondria following TBK1 inhibition, although we did note a ~50% reduction in TAX1BP1 recruitment (Fig 3.7I). These observations suggest that OPTN and potentially TAX1BP1 are regulated by TBK1, while NDP52 may facilitate mitophagy through a TBK1-independent pathway.

3.3.6 Phosphorylation of OPTN S177 is necessary for efficient induction of autophagy following mitochondrial depolarization.

OPTN contains a short, four-residue LIR motif near its N-terminus (positions 178-181), through which it associates with LC3 (Wild et al., 2011). The strength of this association, which reflects the ability of OPTN to recruit autophagic membranes, is augmented by TBK1-mediated phosphorylation of a serine residue just before the LIR, at position S177 (Wild et al., 2011). We thus examined the extent to which phosphorylation of OPTN at S177 influenced the ability of this receptor to mediate autophagosome formation around depolarized mitochondria.

In HeLa cells transfected with Halo-OPTN, Parkin, Mito-DsRed2, and GFP-LC3, 58% of mitochondria were positive for OPTN 90 min after depolarization with CCCP (Fig. 3.8A,D). Autophagosomes effectively formed around these OPTN-positive mitochondria, as 37% were also positive for LC3 (Fig. 3.8F). We then used site-directed mutagenesis to generate a Halo-OPTN construct that could not be phosphorylated at S177 (S177A). Cells were transfected with Halo-OPTN-S177A along with untagged Parkin, Mito-DsRed2, and GFP-LC3. Prior to CCCP treatment, cells expressing the S177A mutant were
indistinguishable from cells expressing wild type OPTN. CCCP treatment induced the translocation of OPTN-S177A to fragmented mitochondria within 20-30 min. At 90 min post-CCCP, OPTN-S177A was uniformly recruited to 75% of the total mitochondrial population (Fig. 3.8B,D). Recruitment of OPTN-S177A to depolarized mitochondria was even more effective than that of wild type OPTN (Fig. 3.8D). Strikingly, however, only 8.1% of OPTN-S177A-positive mitochondria co-recruited LC3 (Fig. 3.8E). This dramatic decrease in LC3 recruitment indicates that phosphorylation of OPTN at S177 is necessary for efficient engulfment of depolarized mitochondria by LC3-positive autophagosomes. Interestingly, HeLa cells expressing OPTN-S177A still robustly recruited TBK1 to depolarized mitochondria (Fig. 3.8S6A) indicating that recruitment of TBK1 to depolarized mitochondria is not dependent on OPTN S177 phosphorylation.

We next asked whether expression of a constitutively active, phosphomimetic form of OPTN would enhance CCCP-induced mitophagy. Cells expressing the phosphomimetic construct Halo-OPTN-S177E as well as Parkin, Mito-DsRed2, and GFP-LC3 appeared phenotypically normal prior to addition of CCCP. Addition of CCCP induced robust recruitment of OPTN-S177E to depolarized mitochondria (Fig. 3.8C,D) and clear formation of LC3-positive autophagosomes around the OPTN-positive mitochondria (Fig. 3.8F). However, we did not see an upregulation of either OPTN or LC3 recruitment to mitochondria in comparison to cells expressing wild type OPTN (Fig. 3.8D-F), suggesting that OPTN is efficiently phosphorylated in CCCP-treated HeLa cells. Together, these results indicate that activation of OPTN through S177 phosphorylation is essential for its ability to function as an autophagy receptor for depolarized mitochondria.
3.3.7 ALS-associated OPTN and TBK1 mutants interfere with mitophagy of depolarized mitochondria

In the last several years, multiple mutations in both OPTN and TBK1 have been identified in patients with ALS and FTD (Cady et al., 2015; Freischmidt et al., 2015; Ito et al., 2011; Maruyama and Kawakami, 2013; Pottier et al., 2015; Weishaupt et al., 2013). We therefore examined whether these disease-associated mutations interfere with efficient mitophagy. We depleted endogenous TBK1 with an siRNA specific for the 3' UTR of TBK1 (leading to a 75% reduction in endogenous levels of TBK1 (Fig. 3.S7 A,B)), and transfected cells with Halo-OPTN, Parkin, Mito-DsRed2, and GFP-LC3. Depletion of TBK1 resulted in a significant decrease in LC3-positive mitochondria at 90 min post-CCCP as compared to cells transfected with a scrambled siRNA (Fig. 3.9A). This mitophagy defect was rescued by expression of WT-TBK1, but not by an ALS-associated E696K-TBK1 mutant (Fig. 3.9A,B). E696K-TBK1 did not associate with OPTN, and failed to translocate to depolarized mitochondria 90 min after CCCP treatment (Fig. 3.9B).

Next we asked whether ALS-associated OPTN mutations similarly interfered with recruitment of LC3 to depolarized mitochondria. We examined three ALS associated OPTN mutants: 1) OPTN-E478G (ubiquitin binding domain mutant), 2) OPTN-R96L (coiled-coil domain 1 mutant), and 3) OPTN-Q398X (truncation mutant entirely lacking ubiquitin domain). As previously shown, expression of WT OPTN in Parkin-expressing cells results in a robust enhancement of mitophagy at 90 min post-CCCP. To examine whether these ALS-associated OPTN mutants could similarly enhance mitophagy, we transfected cells with untagged parkin, mCh-LC3, Mito-SNAP, and either GFP-OPTN,
GFP-E478G-OPTN, GFP-Q398X-OPTN, or GFP-R96L OPTN. Both E478G-OPTN and Q398X-OPTN remained cytosolic at 90 min post-CCCP, failing to translocate to depolarized mitochondria and enhance mitophagy (Fig. 3.9C,D). In contrast, both wildtype and R96L-OPTN translocated to depolarized, fragmented mitochondria, inducing equivalent levels of autophagosome formation (Fig. 3.9C,D). Thus, expression of some but not all ALS-linked mutations in OPTN can disrupt efficient mitophagy.

3.4 Discussion

In this study we used live cell imaging to examine the dynamics of autophagy receptors and their regulation during selective mitophagy. Previously, we found that the ALS-associated receptor OPTN mediates the autophagic engulfment of Parkin-positive mitochondria (Wong & Holzbaur, 2014). Here, we examined the regulation of OPTN by TBK1, an upstream kinase also linked to ALS. We find that TBK1 is dynamically recruited with OPTN to depolarized, Parkin-positive mitochondria. Inhibition of TBK1 does not hinder the initial recruitment of OPTN, but does dramatically inhibit the assembly of autophagic membranes around depolarized mitochondria. As knockdown of TBK1 resulted in a more significant inhibition of LC3 recruitment than we observed following OPTN knockdown, our observations support a model in which multiple receptors contribute to the mitophagy of damaged mitochondria. Specifically, we found that two other autophagy receptors, NDP52 and TAX1BP1, are recruited with similar kinetics to depolarized, Parkin-positive mitochondria. Overexpression of any of these three receptors enhances the formation of autophagosomes around depolarized mitochondria, suggesting that cellular expression levels of these receptors may be limiting. However, we noted that
NDP52 depletion had little effect on the kinetics of sequestration of depolarized mitochondria over a time frame of up to 3 hours, in contrast to our observations on OPTN depletion. Thus, while there may be functional compensation over longer time frames (Lazarou et al., 2015), kinetically these receptors are not interchangeable.

A recent investigation reported that stable knockout of OPTN in HeLa cells did not have a significant effect on mitochondrial clearance 24h after depolarization (Lazarou et al., 2015). Using the same knockout line, we observed a modest but significant 20% decrease in the formation of mitochondrial autophagosomes at 3h post-CCCP. Thus, loss of OPTN affects the efficiency of autophagosome formation in both knockdown and knockout paradigms. However, at longer time points, both OPTN knockdown and knockout HeLa cells can effectively clear damaged mitochondria, likely through recruitment of alternative receptors such as NDP52. This question has been addressed in detail in bacterial autophagy, or xenophagy, where the interplay amongst autophagy receptors is essential for efficient autophagosomal engulfment of invasive pathogens (Boyle and Randow, 2013). Here we show that multiple receptors are recruited to depolarized mitochondria with similar kinetics, yet these receptors are only partially redundant in effecting efficient mitochondrial engulfment.

Studies on xenophagy have also identified a key regulatory role for TBK1, a serine/threonine kinase that has been demonstrated to interact with OPTN, NDP52, and TAX1BP1 (Thurston et al., 2009; Tumbarello et al., 2015; Wild et al., 2011). Here, we found that OPTN and TBK1 translocate to depolarized mitochondria with similar kinetics. OPTN likely shuttles TBK1 to damaged mitochondria, as depletion of OPTN or expression of a TBK1 mutant unable to bind OPTN eliminates recruitment of TBK1 to depolarized mitochondria.
mitochondria. However, recruitment of OPTN is not dependent on TBK1 expression or TBK1 activity; similarly, TAX1BP1 and NDP52 are also recruited to depolarized mitochondria when TBK1 is inhibited.

Though inhibition of TBK1 does not interfere with the initial recruitment of OPTN to mitochondria, it does block the stabilization of OPTN and the concomitant recruitment of LC3 to depolarized mitochondria, suggesting that mitochondrially localized OPTN must be subsequently activated by TBK1 in order to function as an autophagy receptor. In contrast, NDP52 can facilitate the formation of autophagosomes on depolarized mitochondria, even in the absence of TBK1. This finding suggests that OPTN and TAX1BP1 act in the same pathway downstream of TBK1, while NDP52 may function in a parallel mitophagy pathway less dependent on TBK1 (Fig. 3.10), as initially suggested by Lazarou et al. (2015).

In these studies, we induced mitophagy using several approaches, including depolarization by the mitochondrial uncoupler CCCP, inhibition of the electron transport chain by Antimycin and Oligomycin, and reactive oxygen species production by activation of a localized photosensitizer (MitoKR). Irrespective of the upstream damage, we observed very similar recruitment kinetics for the autophagy receptors, and consistent timing of LC3 recruitment to form autophagosomes around depolarized mitochondria. Typically, OPTN, NDP52, and TAX1BP1 display a weak pattern of recruitment to depolarized mitochondria 10-15 min after recruitment of Parkin. Starting 10 min after this weak recruitment, a small subset of mitochondria display enhanced receptor intensity coupled to more stable receptor binding, coincident with the recruitment of LC3 puncta.
Over the next ~5 min, LC3 puncta develop into spherical structures entirely circumscribing depolarized, Parkin-positive mitochondria.

By 90 min after CCCP-induced depolarization, nearly every mitochondrion is Parkin-positive, more than half show clear autophagy receptor rings, but only 25% are effectively sequestered by LC3-positive autophagosomes. Even at longer time points, such as 5 or 8h post-CCCP, a number of OPTN-positive mitochondria can still be clearly seen outside of autophagosomes. The variability in efficiency with which individual mitochondria are targeted to autophagosomes after cell-wide depolarization may be limited by several factors, including expression levels of autophagy receptors, the relative activation of upstream regulatory signals, such as TBK1, and possibly the availability of autophagic membrane sources and thus the rate of autophagosome biogenesis. However, the sooner depolarized mitochondria are quarantined from the rest of the cell by autophagosomes, the less damage they are likely to cause. Therefore, the rate at which damaged mitochondria are effectively engulfed and sequestered by an isolation membrane may be among the most important aspects of mitophagy.

A recent exome sequencing study identified significantly higher rates of TBK1 polymorphisms in individuals with ALS (Cirulli et al., 2015). Another study linked loss-of-function TBK1 mutations to both ALS and frontotemporal dementia (Freischmidt et al., 2015). Finally, mutations in both TBK1 and OPTN are causal for both ALS and glaucoma (Pottier et al., 2015; Sirohi et al., 2015). In this study we observed that the ALS-associated mutants E696K-TBK1, E478G-OPTN, and Q398X-OPTN all interfere with efficient autophagic engulfment of damaged mitochondria. However, we did not observe mitophagy defects in cells expressing R96L-OPTN. Thus, disordered mitophagy may
contribute to the pathophysiology of ALS and FTD, but further work is required to fully establish this point. Specifically, slower and/or less effective mitophagy might lead to the accumulation of damaged mitochondria and subsequent cytotoxic stress. Consistent with the late onset nature of both ALS and glaucoma, other receptors likely compensate to some degree, but over time the decreased efficiency caused by TBK1 or OPTN mutations may be sufficient to lead to enhanced neurodegeneration.

It is not yet clear why mutations in ubiquitously expressed proteins result in selective degeneration of specific neuronal populations. Perhaps expression levels of compensatory mitophagy receptors such as NDP52 are limiting in affected cells. Consistent with this hypothesis, levels of NDP52 protein are comparatively low in human brain lysates (Lazarou et al., 2015). Alternatively, the effects of dysregulated mitochondrial quality control might simply manifest in specific neuronal populations earlier and to a greater extent than other cell types. Motor neurons and retinal ganglion cells affected in ALS and glaucoma are characterized by extended axonal processes; whether damaged mitochondria occupying distal axonal regions are subject to the same quality control mechanisms as those near the cell body remains unclear (Ashrafi et al., 2014; Cai et al., 2011). It is also a distinct possibility that mutations in TBK1 and OPTN contribute to disease through other mechanisms, including aberrant protein aggregate autophagy (aggrephagy) or neuroinflammatory pathways; further work will be required to address these possibilities.
3.5 Methods

Constructs, siRNAs, and Antibodies. Constructs used include: Mito-DsRed2 (gift from Dr. T. Schwarz), recloned into SNAP-Tag (NEB) and psBFP2-C1 (Addgene), TBK1 (Addgene #23851) recloned into pSNAPf vector, pEGFP-LC3 (gift from Dr. T. Yoshimori) recloned with pmCherry (Takara Bio inc.), YFP-Parkin and mCherry-Parkin (gifts from Dr. R. Youle), untagged Parkin, pEGFP-OPTN and pEGFP-E478G-OPTN (gifts from Dr. I. Dikic) recloned with pmCherry (Takara Bio inc.) and HaloTag (Promega), R96L-EGFP (Addgene #68846), Q398X-EGFP (Addgene #68849), HaloTag-NDP52 (Promega) recloned into pEGFP, HaloTag-TAX1BP1 (Promega) recloned into pEGFP (Promega), and pKillerRed-dMito (Evrogen). Site directed mutagenesis was used to generate mCherry-Parkin-T240R, HaloTag-OPN-S177A, HaloTag-OPN-S177E, SNAP-S172A-TBK1, and SNAP-E696K-TBK1. HaloTag constructs were labeled with either HaloTag TMR ligand (Promega, G821) or Silicon-Rhodamine-Halo ligand (gift from Dr. L. Reymond and Dr. K. Johnsson). SNAP-Tag constructs were labeled with SNAP-Cell 647-SiR (NEB, S9102S) or SNAP-Cell 430 (NEB, S9109S). siRNA oligos used include: OPTN siRNA 1 (Dharmacon ON-TARGET plus SMART pool, OPTN), OPTN siRNA 2 (Dharmacon, 5'-CCACCAGCTGAAAGAAGCC-3'), TBK1 siRNA (h) (Santa Cruz, sc-39058), TBK1 3' UTR siRNA (Dharmacon) SignalSilence NDP52 siRNA 1 (Cell Signaling, 8964S) and fluorescent ON-Target plus Non-targeting siRNA #1 with 5' Cy5 (Dharmacon). Antibodies used include: Optineurin (abcam, ab23666), TBK1/NAK (abcam, ab40676), NDP52 (abcam, ab68588), GAPDH (abcam, ab9494), and actin (Calbiochem, MAB1501).

Cell Culture, Reagents, and Live Imaging. HeLa Cells were maintained in DMEM (Corning) with 10% FBS and 1% Glutamax, and kept at 37°C in a 5% CO2 incubator. 24 hours before transfection, cells were plated on uncoated 35 mm glass-bottom dishes
DNA constructs were transfected 18-24 h before imaging using FuGene 6 (Promega, E2691). SiRNAs were transfected 48h prior to imaging with Lipofectamine RNAiMAX (Thermo Fisher, 13778030). Transfection efficiency for both DNA and RNA was typically >90%. To assess mitochondrial membrane potential, cells were loaded with 30 nM TMRE (Life Technologies, T-669) for 30 min, followed by 2x washes in complete media. SNAP and HaloTag ligands were applied at 2.5 µM. SNAP-tag ligand was applied for 30 minutes, followed by 2x washes and a 30 min washout. Halo-tag was applied for 15 minutes, followed by 2x washes in complete media. OPTN, NDP52, and OPTN/NDP52 knockout HeLa cell lines (gifts from Dr. R. Youle) were maintained in identical conditions as wildtype HeLa cell lines. Prior to imaging, HeLa cell culture media was replaced with imaging media (DMEM without phenol red +25 mM HEPES (Corning), with 10% FBS, and 1% Glutamax). Cells were then transferred to a Nikon Eclipse Ti Microscope housed within a 37°C environment chamber and imaged at 100x (Aprochromat, 1.49 NA oil immersion objective) using an UltraView Vox spinning disk confocal imaging system (Perkin Elmer). Mitochondrial depolarization was induced by bath application of 20 µM cyanide m-chlorophenyl hydrazone (Sigma-Aldrich, C2759) or 10 µM Antimycin A (Sigma-Aldrich, A8674) with 10 µM Oligomycin (pool of A, B, and C, Sigma-Aldrich, 04876). Mitophagy in cells expressing both Parkin and autophagy receptors was assessed at 90 min post-CCCP, while cells expressing Parkin alone were analyzed at 180 min post-CCCP. For TBK1 inhibition experiments, cells were pretreated with 1 µM BX795 (Calbiochem, 204001) for 1h prior to CCCP treatment. Still frame images, z-stacks, and time-lapse movies were acquired using Volocity image acquisition software. Focal mitochondrial damage was induced through photoirradiation of MitoKiller Red by 561 nm laser light. Specifically, two ~15x15 µm regions were bleached with a 561 nm laser for 100 cycles lasting no longer than 3 min in total. For FRAP experiments,
HaloTag-OPTN rings at 90 min post-CCCP were bleached with a 640 nm laser at 100% for 100 cycles at 1 ms/cycle. Standard immunoblot analysis of whole cell lysates confirmed NDP52 and TBK1 knockdowns (Fig. S4E,G).

Image Analysis and Statistics. Mito-DsRed2 fragments, GFP-LC3 rings, HaloTag-OPTN rings, HaloTag-NDP52 rings, and HaloTag-TAX1BP1 rings were manually counted in ImageJ (NIH) and Volocity (PerkinElmer). Only clearly defined mitochondrially localized rings were counted. 2-pixel wide line scans through mitochondria were generated in ImageJ, normalized in Excel (Microsoft), and graphed in Prism (GraphPad). Autophagy receptor and LC3 ring intensity was calculated as the average intensity of the two highest peak values along line scans and normalized per cell and condition. TMRE and SNAP-Tag-Mito fluorescence intensity was calculated as the mean intensity along 2-pixel wide line scans. Global Pearson’s Correlations for bleached and unbleached regions were generated in Volocity. Z-projections and time-lapse movies were generated in ImageJ, and 3D renderings were produced in Volocity. Statistical analyses were carried out on data sets consisting of at least three independent experiments, using either a two-tailed unpaired Student’s t-test comparing two groups, one-way Analysis of Variance (ANOVA) with Tukey’s multiple comparison test when comparing more than two groups, or two-way ANOVA with Tukey’s multiple comparison test when comparing the main effects of autophagy receptor (OPTN, NDP52, TAX1BP1, or endogenous) and treatment (DMSO or BX795). Error bars = mean ± S.E.M. Images and figures were prepared in Illustrator (Adobe).
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3.6 Figures

3.6.1 Fig 3.1

Figure 3.1. OPTN, NDP52, and TAX1BP1 are dynamically recruited to depolarized mitochondria after CCCP treatment to initiate autophagosome formation. (A) Representative confocal images showing localization of Mito-DsRed2 (blue) and GFP-LC3 (green) in Parkin-expressing HeLa cells treated with either DMSO or 20 µM CCCP. Arrows indicate mitochondria engulfed by autophagosomes. (B) Co-expression of OPTN with Parkin enhances mitophagy at 180 min post-CCCP. Depletion of endogenous OPTN significantly reduces mitophagy at this time point. (C-E) HeLa cells expressing untagged Parkin, Mito-DsRed2, GFP-LC3, and either (C) Halo-OPTN, (D) Halo-NDP52, or (E) Halo-TAX1BP1 were treated with 20 µM CCCP for 90 min. (C) At 90 min post-CCCP, OPTN and LC3 were enriched on the outer surface of fragmented mitochondria (yellow arrowhead). Line-scan analysis reveals overlapping OPTN and LC3 local maxima around peak mitochondria (mito) fluorescence. (D) Cells expressing Halo-NDP52 exhibited stable NDP52 and LC3 recruitment to depolarized mitochondria at 90 min post-CCCP (yellow arrowhead). Corresponding line scan indicating NDP52/LC3 colocalization around indicated mitochondria. (E) Halo-TAX1BP1 also colocalized with LC3 around depolarized mitochondria at 90 min post-CCCP. Line scan shows corresponding peaks of normalized LC3 and TAX1BP1 intensity. (F) OPTN, NDP52, and TAX1BP1 are robustly recruited to mitochondria after 90 min CCCP treatment. 

Expression of each receptor with Parkin enhances mitophagy at 90 min post-CCCP as compared to cells expressing Parkin alone. Scale bar (A, C-E, full size) 10 µm, (A, C-E, zoom) 2.5 µm. Data were collected from 21-70 cells from at least three independent experiments. Bars represent mean ± S.E.M., *** p < .001.
3.6.2 Fig 3.2.

Figure 3.2. Focal ROS production induces translocation of LC3 and autophagy receptors to damaged mitochondria. (A-C, left) Maximum intensity projections of HeLa cells expressing Parkin, Mito-SNAP (blue), GFP-LC3 (green), MitoKR (red) and either (A) Halo-OPTN, (B) Halo-NDP52, or (C) Halo-TAX1BP1 (magenta) 90 min after activation of MitoKR by 561 nm laser light. (A) At 90 min post-bleach, OPTN and LC3 selectively translocate to fragmented mitochondria (arrow) within the bleach window, but not to unbleached mitochondria on the opposite side of the cell. (B) 90 min post MitoKR activation, NDP52 and LC3 are robustly recruited to bleached mitochondria (arrow). (C) TAX1BP1 and LC3 are recruited to damaged mitochondria within the bleach windows 90 min after MitoKR activation (arrow). Scale bar (A-C, full size) 10 µm, (A-C, zoom) 2.5 µm.
Figure 3.3. Autophagy receptor recruitment kinetics. (A-C) Confocal time series showing recruitment of autophagy receptors and LC3 to damaged mitochondria after MitoKR activation. OPTN (A), NDP52 (B) and TAX1BP1 (C) begin to accumulate on damaged mitochondria within 30 min. By 45 min, LC3-rings can be visualized forming on receptor-positive mitochondria. At 75 min post-MitoKR there is significant colocalization of each autophagy receptor with LC3 in the bleached region, but not in distal unbleached regions. (D-F) Global Pearson's correlation of each receptor to LC3 increases over time within the bleach window, but not in unbleached regions. Scale bar (A-C) 5 µm. Vertical dashed lines indicate bleach.
3.6.4 Fig 3.4.

Figure 3.4. Autophagy receptors are recruited and stabilized on damaged mitochondria in a two-step process. (A,C) Prior to LC3 binding, OPTN is weakly recruited to fragmented mitochondria, gradually increasing in intensity over time. When LC3 is recruited ~15 min later, OPTN intensity markedly increases, before stabilizing with the formed autophagosome at 25 min post-initial recruitment. (B,D) As with OPTN, NDP52 is initially weakly recruited to fragmented mitochondria. NDP52 binding to fragmented mitochondria is enhanced as LC3 rings form, and stabilized ~25 min after initial recruitment. Dashed lines indicate steps of autophagy receptor recruitment. (E-G) At 90 min post-CCCP, mitochondrially-localized autophagy receptors display significantly higher normalized intensity on LC3-positive mitochondria as compared to LC3-negative mitochondria. (H) At this time point, mitochondrially-localized Parkin intensity is not affected by autophagosome formation. (I-K) To probe the stability of OPTN on mitochondria with or without LC3, OPTN rings on depolarized mitochondria were bleached with 640 nm laser light. (I) Confocal time series shows OPTN fluorescence recovery on LC3-negative mitochondria (red arrow) but not LC3-positive mitochondria (green arrow) after photobleaching. (J) Magnified view of boxed region in I shows clear recovery of OPTN signal intensity 5 min after 640 nm laser bleaching (red arrow). (K) Magnified view of boxed region in I shows little recovery of OPTN signal 5 min post-bleach (green open arrow). (L) LC3-negative mitochondria display significantly higher fluorescence recovery after photobleaching. Scale bar (I) 5 μm, (A,B,J,K) 1 μm. (E-H) n=50 mitochondria from 5 different cells per comparison. Error bars represent mean ± S.E.M. (L) Fluorescence recovery of OPTN on 3 mitochondria per condition were fit to a single exponential association model. ***p<.001.
Figure 3.5. OPTN depletion blocks efficient mitophagy. (A) Representative confocal images of mitochondria-positive autophagosomes at 180 min post-CCCP in Parkin-expressing cells. (B) Depletion of OPTN significantly impairs autophagosome formation at 180 min post-CCCP. In contrast, knockdown of NDP52 does not affect LC3 recruitment to depolarized mitochondria at this time point. Scale bar (A-B full size) 10 μm, (A-B zoom), 2.5 μm. Data were collected from 29-49 cells from at least three independent experiments. Bars represent mean ± S.E.M. *** $p < .0001$, n.s. = not significant.
TBK1 is co-recruited with OPTN to depolarized mitochondria. (A) Time series tracking recruitment of SNAP-TBK1 (green) and GFP-OPTN (red) to depolarized mitochondria in a Parkin-expressing cell. Time stamp indicates elapsed time since addition of CCCP. Immediately after CCCP treatment, OPTN and TBK1 are largely cytosolic, but colocalize in small puncta (white arrowheads, top panel). At 15 min post-CCCP, both OPTN and TBK1 begin to appear on fragmented mitochondria (yellow arrowheads). (B) Magnified view of boxed region in panel A with corresponding line scan indicating TBK1/OPTN colocalization on an individual mitochondrion. (C) Confocal z-stack showing recruitment OPTN and TBK1 to fragmented mitochondria (yellow arrowheads) 90 min post-CCCP treatment. (D) Depletion of OPTN eliminates TBK1 recruitment to depolarized mitochondria. (E) Overexpression of OPTN enhances TBK1 recruitment to parkin-positive mitochondria at 90 min post-CCCP. (F) Endogenous TBK1 was depleted and rescued with either wildtype TBK1 (WT, top panel) or an ALS-linked TBK1 mutant unable to bind OPTN (E696K, bottom panel). WT-TBK1 was clearly enriched on fragmented mitochondria after CCCP treatment, while E696K-TBK1 remained cytosolic. Scale bar (C, full size) 10 μm, (A,D,E) 5 μm, (F, C zoom) 2.5 μm, (B) 0.5 μm.
3.6.7 Fig 3.7.

Figure 3.7. TBK1 is required for efficient autophagic engulfment of depolarized mitochondria. (A-C) HeLa cells expressing untagged-Parkin, Mito-DsRed2 (blue), Halo-OPTN (red), and GFP-LC3 (green). (A) Representative confocal image of DMSO pre-treated HeLa cell at 90 min post-CCCP with corresponding line scan. OPTN and LC3 stably localize on the surface of damaged mitochondria (yellow arrowhead). (B) Pre-treatment with 1 µM BX795 significantly attenuates LC3, but not OPTN recruitment to depolarized mitochondria (red arrow). Corresponding line scan indicates increased OPTN, but not LC3 fluorescence around peak mito intensity. (C) TBK1 depletion blocks association of LC3 with fragmented mitochondria at 90 min post-CCCP (red arrowhead). Line scan indicating OPTN but not LC3 recruitment to fragmented mitochondria. (D) Inhibition of TBK1 by BX795 increases the proportion of cellular mitochondria that recruit OPTN, while knockdown of TBK1 decreases OPTN-positive mitochondria. (E-F) Both TBK1 knockdown and inhibition by BX795 significantly impair autophagosome formation on OPTN-positive mitochondria at 90 min post-CCCP. (G) In Parkin-expressing cells, depletion or inhibition of TBK1 interferes with mitophagy at 90 min post-CCCP. (H) Co-expression of Parkin with either OPTN or TAX1BP1 significantly enhances mitophagy at 90 min post-CCCP in control cells, but not in cells treated with a TBK1 inhibitor. In contrast, co-expression of Parkin with NDP52 robustly enhances mitophagy independent of TBK1 activity. (I) TAX1BP1 recruitment to depolarized mitochondria is diminished in cells pretreated with BX795. (J) NDP52-positive mitochondria, but not OPTN- or TAX1BP1-positive mitochondria are efficiently engulfed by autophagosomes after TBK1 inhibition. Scale bar (A-C, full size) 10 µm, (A-C, zoom), 2.5 µm. Data were collected from 19-61 cells from at least three independent experiments. Bars represent mean ± S.E.M. *** p < .001.
OPTN S177 phosphorylation is required for efficient mitophagy. HeLa cells expressing untagged-Parkin, Mito-DsRed2, GFP-LC3, and either (A) wildtype OPTN, (B) OPTN-S177A, or (C) OPTN-S177E were treated with 20 µM CCCP for 90 min. (A) At 90m post-CCCP, wildtype OPTN and LC3 are enriched on the outer surface of mitochondria (yellow arrowhead). Line scan analysis through indicated mitochondrion shows overlapping OPTN and LC3 local maxima. (B) Cells expressing OPTN-S177A show clear recruitment of phosphodeficient OPTN to fragmented mitochondria, but fail to efficiently recruit LC3 after CCCP treatment (red arrowhead). Corresponding line scan indicating S177A, but not LC3, localization to mito. (C) Phosphomimetic OPTN-S177E, like wildtype, translocates with LC3 to the surface of mitochondria after CCCP treatment (yellow arrowhead). Line scan showing S177E/LC3 colocalization around mito. (D-F) Phosphodeficient OPTN is robustly recruited to mitochondria after CCCP treatment as compared to wildtype OPTN or S177E, but blocks recruitment of LC3. Scale Bar (A-C, full size) 10µm, (A-C, zoom) 2.5µm. Data were collected from 25-41 cells from at least three independent experiments. Bars represent mean ± S.E.M, *p<.05, **p<.001.
Figure 3.9 ALS-associated TBK1 and OPTN mutants block mitophagy.

(A) Representative confocal images of Parkin/OPTN-expressing HeLa cells in which endogenous TBK1 has been knocked down and rescued with either WT-TBK1 or E696K-TBK1. (B) TBK1 depletion significantly blocks mitophagy at 90 min post-CCCP as compared to scrambled control. Expression of WT-TBK1, but not ALS-linked E696K-TBK1 rescues this mitophagy defect. (C) Representative confocal images of HeLa cells expressing untagged parkin, mCherry-LC3 (green), Mito-SNAP (blue), and either GFP-OPTN, GFP-R96L-OPTN, GFP-E478G-OPTN, or GFP-Q398X-OPTN (red) at 90 min post-CCCP. While WT-OPTN and R96L-OPTN colocalize with LC3 on fragmented mitochondria after CCCP treatment (yellow arrows), both Q398X-OPTN and E478G-OPTN remain cytosolic. (D) At 90 min post-CCCP, R96L-OPTN and WT-OPTN enhance mitophagy, while cells expressing Q398X-OPTN and E478G-OPTN show significantly lower levels of LC3-positive mitochondria. Scale Bar (A,B full size) 10µm, (A,B zoom) 2.5µm. Data were collected from 21-33 cells from at least three independent experiments. Bars represent mean ± S.E.M., ***p<.001, n.s. = not significant.
3.6.10 Fig 3.10

Figure 3.10. Model depicting autophagy receptor recruitment to depolarized mitochondria over time. 1) Prior to a depolarizing injury, mitochondria are elongated and motile. 2) Within 15 min of injury, damaged mitochondria recruit PINK1 and Parkin, resulting in phospho-ubiquitination of outer membrane proteins and mitochondrial fragmentation. 3) 25 min after initial damage, and within ~10 min of first Parkin recruitment, autophagy receptors OPTN, TAX1BP1, and NDP52 are weakly recruited to the surface of fragmented mitochondria. OPTN shuttles its upstream kinase TBK1 to the mitochondrial surface. Expression of the Parkinson’s disease associated T240R-Parkin mutant blocks recruitment of all three receptors. Additionally, ALS-linked E478G- and Q398X-OPTN mutants are not recruited to ubiquitinated mitochondria. 4) Within 5-10 min of weak autophagy receptor recruitment, a small number of mitochondria display enhanced autophagy receptor recruitment and stabilization, coincident with the recruitment of the autophagosome membrane protein LC3. This step is dependent on TBK1 phosphorylation of OPTN S177, and is blocked in cells expressing ALS-linked E696K-TBK1. While OPTN and TAX1BP1 depend on TBK1 activity in this pathway, NDP52 can stabilize on mitochondria and facilitate autophagic engulfment of mitochondria even in the absence of TBK1. Thus high levels of NDP52 can compensate for OPTN or TBK1 loss of function. 5) By 40-45 min after the initial damage, fully formed autophagosomes develop around mitochondria, effectively sequestering the damaged organelles from the surrounding cytosol. Bolded mutants have been linked to neurodegenerative disease.
3.6.11 Fig 3.S1

Figure 3.S1. (A) HeLa cell loaded with 30 nM tetramethyl rhodamine ethyl ester (TMRE) displays high fluorescence intensity prior to addition of 20 µM CCCP. Within two min, the majority of mitochondria have been depolarized, resulting in decreased TMRE fluorescence. By 6 min, all mitochondria have expelled the TMRE dye. (B) Confocal time series showing individual mitochondria labeled with TMRE and Mito-SNAP, a genetically encoded mitochondrial marker that is insensitive to membrane potential. Prior to CCCP treatment, mitochondria are double labeled with Mito-SNAP and TMRE, and YFP-Parkin is cytosolic. Within 1 min of CCCP treatment, the mitochondria can still be visualized by Mito-SNAP, but TMRE is expelled from the mitochondria (white arrowhead). 40 min later, Parkin is recruited to the depolarized mitochondria. (C) Normalized intensity of Mito-SNAP and TMRE after 20 µM CCCP addition (dashed line). (D) 180 min CCCP treatment fails to induce mitophagy in HeLa cells due to low levels of endogenous Parkin. Scale bar (A, D full size) 10 µm, (B, D, zoom) 2.5 µm.
Figure 3.52. (A) Halo-TAX1BP1, mCherry-OPTN, and GFP-NDP52 colocalize on depolarized mitochondria 90 min post-CCCP in Parkin-expressing HeLa cells. (B-C) Recruitment of either NDP52 or TAX1BP1 to depolarized mitochondria is dependent on Parkin and blocked by expression of the Parkinson’s disease associated T240R Parkin mutant. HeLa cells transfected with Mito-DsRed2, mCherry-Parkin-T240R, and either (B) Halo-NDP52 or (C) Halo-TAX1BP1. At 60 min post-CCCP, Parkin-T240R is recruited to depolarized mitochondria, but NDP52 and TAX1BP1 remain largely cytosolic. (D) In the absence of exogenous parkin, NDP52 and LC3 fail to translocate to depolarized mitochondria at 90 min post-CCCP. Scale bar (B-D, full size) 10 µm, (A) 5 µm, (B-D, zoom) 2.5 µm.
Figure 3.33. (A) Mitochondria double labeled with Mito-SNAP (blue) and the photosensitizer MitoKR (red). Before bleach (top row), Mito-SNAP and MitoKR signals appear identical. After sustained laser illumination with 561 nm light (bottom row), the MitoKR signal disappears, but Mito-SNAP still clearly labels mitochondria. (B) Confocal time series showing recruitment of YFP-Parkin within 12-15 min of MitoKR photobleaching. Scale (A-B) 10 µm.
3.6.14 Fig 3.S4

Figure 3.S4. (A) Treatment with 20 nM siRNA targeting NDP52 resulted in >95% depletion of endogenous NDP52. (B) Effects of OPTN depletion by transient knockdown and knockout. (B) In the HeLa cell line in standard use in our lab (HeLa Line 1), transient knockdown of OPTN with either of two siRNA oligos significantly reduces the engulfment of mitochondria into autophagosomes 3h post-CCCP treatment. We also used live cell microscopy to examine mitophagy in OPTN knockout cells from Lazarou et al. (2015). At 180 min post-CCCP, OPTN knockout cells display significantly decreased mitophagy as compared to the HeLa cell line from which they were generated (HeLa Line 2). While knockout of NDP52 did not induce a significant defect in mitophagy, we did observe a more pronounced defect in the double knockout cells, suggesting that NDP52 may only partially compensate for loss of OPTN at shorter time points following mitochondrial depolarization (180 min as shown here), but can compensate more fully at longer time points (24 hr; Lazarou 2015). Of note, there is a significant difference in baseline mitophagy levels 180 min post-CCCP between the two parental cell lines (HeLa line 1 and HeLa line 2), which may result from differences in endogenous expression levels of autophagy receptors (C). Bars represent mean ± S.E.M. Data were collected from at least three independent experiments *p<.05, ***p<.001, n.s.= not significant.
Figure 3.S5. (A,B) Inhibition of TBK1 by 1 h pretreatment with 1 µM BX795 or expression of a phosphodeficient S172A-TBK1 mutant does not block TBK1 recruitment to depolarized mitochondria at 90 min post-CCCP. (C) Inhibition of TBK1 blocks GFP-LC3 recruitment at 90 min post-CCCP, but does not block upstream recruitment of mCherry-Parkin or Halo-OPTN. (D, top row) 90 min treatment with 10 µM Antimycin along with 10 µM Oligomycin induces recruitment of LC3 and OPTN to fragmented mitochondria. (D, bottom) LC3 recruitment, but not OPTN recruitment is blocked by pretreatment with 1 µM BX795. (E) Immunoblot of TBK1 and actin loading control. Treatment of HeLa cells with 20 nM TBK1 siRNA for 48h results in a > 95% reduction in TBK1 protein levels. (F) At 90 min post-CCCP, the percent of mitochondria positive for LC3 in Parkin/OPTN expressing cells is not affected by DMSO (vehicle) treatment or expression of a scrambled siRNA. (G) Treatment of cells with 100 nM NDP52 siRNA for 48h resulted in a >95% reduction in NDP52 protein levels as compared to cells treated with a scrambled siRNA. Scale bar (D, full size) 10 µm, (A, B, C, D zoom), 5 µm. Bars represent mean ± S.E.M.
3.6.16 Fig 3.S6

Figure 3.S6: (A) Expression of S177A-OPTN does not interfere with recruitment of TBK1 to depolarized mitochondria at 90 min post-CCC P.

3.6.17 Fig 3.S7

Figure 3.S7. (A-B) Treatment of cells with 20 nM siRNA targeted to the 3’ UTR of TBK1 resulted in a >75% reduction in TBK1 protein levels as compared to cells treated with a scrambled siRNA. Data were collected from three independent experiments. Bars represent mean ± S.E.M.
CHAPTER 4: SPATIOTEMPORAL DYNAMICS OF AUTOPHAGY RECEPTORS IN SELECTIVE MITOPHAGY


4.1 Abstract
Damaged mitochondria are turned over through a process of selective autophagy termed mitophagy. In mitophagy, unhealthy mitochondria are recognized and ubiquitinated by Parkinson disease-linked proteins PINK1 and PARK2. The subsequent recruitment of ubiquitin-binding autophagy receptors leads in turn to the sequestration of the damaged organelles into LC3-positive phagophores, precursors to autophagosomes. The precise identity of these receptors and how they are regulated has been the focus of considerable attention. Our recent work uses live-cell imaging to explore the dynamics and regulation of autophagy receptor recruitment. Utilizing multiple paradigms to induce mitochondrial damage, we identified the rapid, 2-step recruitment of autophagy receptors OPTN, CALCOCO2/NDP52, and TAX1BP1. All 3 receptors are recruited to damaged mitochondria with similar kinetics; however, only OPTN is necessary for efficient formation of a phagophore sequestering damaged mitochondria from the cytosol. OPTN is co-recruited to damaged mitochondria along with its upstream kinase TBK1. Depletion of OPTN or TBK1, or expression of amyotrophic lateral sclerosis (ALS)-linked mutations in either protein, interfere with efficient autophagic engulfment of depolarized mitochondria. These observations suggest that insufficient autophagy of damaged mitochondria may contribute to neurodegenerative disease.

4.2 Results and discussion
Mitochondrial homeostasis is maintained through several quality control mechanisms including dynamic fission/fusion, the mitochondrial unfolded protein response, and selective mitochondrial autophagy (mitophagy). In one model for selective mitophagy, depolarized mitochondria are identified and ubiquitinated by a feed-forward cascade triggered by the ubiquitin kinase PINK1 and the E3 ubiquitin ligase PARK2. Ubiquitination functions as a signal to target damaged mitochondria to the autophagosome-lysosome degradation system. Specifically, autophagy receptors are recruited via binding to poly-ubiquitin chains, and in turn mediate autophagic engulfment through binding to lipidated MAP1LC3 family proteins on autophagic phagophore membranes. Currently, at least 5 ubiquitin-LC3 binding autophagy receptors have been implicated in selective mitophagy: OPTN (optineurin), CALCOCO2, TAX1BP1, NBR1, and SQSTM1.

OPTN was the first of these receptors demonstrated to function in selective mitophagy. Since then, knockout studies have investigated the effects of depleting the other receptors, either singly or in combination, and have demonstrated the involvement of CALCOCO2 and TAX1BP1 in mitophagy. In contrast, multiple studies suggest that SQSTM1 is not required.

In our most recent work, we compared the kinetics of OPTN, CALCOCO2, and TAX1BP1 recruitment to mitochondria. Using live-cell imaging in HeLa cells, we tracked the spatiotemporal localization of fluorescently labeled autophagy receptors in response to either global mitochondrial depolarization by the protonophore CCCP or regional reactive oxygen species generation by the matrix-targeted photosensitizer MitoKillerRed. Both paradigms induce the rapid recruitment of OPTN, TAX1BP1, and CALCOCO2 to damaged mitochondria.
Within 15 min of PARK2 recruitment and 30 min of mitochondrial damage, all 3 autophagy receptors weakly associate with the outer membrane of fragmenting mitochondria. This association is dependent on mitochondrial ubiquitination, and blocked by expression of a Parkinson disease-associated inactive PARK2 mutant. At 45 min post-insult, enhanced recruitment and stabilization of autophagy receptors is observed on a subset of mitochondria, simultaneous with the formation of LC3-positive autophagosomes around the damaged organelles. At 90 min post-insult, more than 50% of damaged mitochondrial fragments are encircled by autophagy receptors; nearly half of these end up sequestered within autophagosomes.

The efficiency with which phagophores engulf damaged mitochondria may be controlled by a number of factors, including expression levels of key mitophagy proteins such as PARK2 or OPTN. Indeed, we find that overexpression of exogenous CALCOCO2 more than quadruples the percentage of mitochondria in autophagosomes at 90 min post-CCCP treatment. Despite this, depletion of endogenous CALCOCO2 has no effect on autophagic engulfment of mitochondria, indicating that CALCOCO2 is not strictly necessary for mitochondrial autophagy. In contrast, depletion of OPTN more than halves the percentage of damaged mitochondria positive for LC3, indicating that OPTN is necessary for initiation of mitophagy under these conditions.

Mutations in both OPTN and its upstream kinase TBK1 have been identified in several cases of familial ALS. Thus, we investigated the interplay between these 2 ALS-linked proteins in mitophagy. We observed robust corecruitment of TBK1 with OPTN to damaged mitochondria. We hypothesize that OPTN is responsible for recruiting TBK1 to the
mitochondrial surface, as either depletion of OPTN or expression of an OPTN-binding TBK1 mutant interferes with recruitment of the kinase to depolarized mitochondria.

Once recruited, TBK1 phosphorylates OPTN at position S177, immediately adjacent to the LC3-interacting region motif. S177 phosphorylation activates OPTN, facilitating engulfment by LC3-positive phagophores. Inhibition or knockdown of TBK1 stalls mitophagy. Similarly, cells expressing phosphodeficient S177A OPTN efficiently recruit PARK2 and OPTN, but fail to recruit LC3. This failure can be overcome by overexpressing CALCOCO2, further supporting the hypothesis that expression levels of mitophagy receptors are critical for effective sequestration of damaged organelles, especially in response to cell-wide insults.

To investigate whether specific ALS-associated mutations in OPTN or TBK1 interfere with mitophagy, we depleted endogenous TBK1 and compared the extent of rescue by wild-type TBK1 or the ALS-linked TBK1E696K mutant. Whereas wild-type TBK1 fully restores mitophagy, cells expressing the mutant kinase display a profound mitophagy defect. Similarly, expression of 2 ALS-linked OPTN mutants, OPTNE478G and OPTNQ398X significantly impair mitochondrial autophagy. However, we noted that a third ALS-linked mutant, OPTNR96L, successfully facilitates mitophagy in these assays, indicating more work is required to fully explore the links between defective mitophagy and ALS.

Taken together, this work underscores the importance of OPTN and its upstream kinase TBK1 in the initiation of damage-induced mitophagy. We find that multiple receptors are involved in the recognition of ubiquitinated mitochondria; these receptors are differentially regulated and only partially redundant (Fig. 4.1) Finally, we observed that multiple
neurodegenerative disease-associated mutations interfere with the efficiency of autophagic engulfment of damaged mitochondria, including mutations in PARK2, OPTN, and TBK1. Thus, disordered mitophagy may represent a key pathophysiological mechanism common to both Parkinson disease and ALS.

It is important to note, however, that much of the work investigating the PINK1-PARK2 mitophagy pathway has been carried out in immortalized cell lines. Though immortalized cells represent excellent model systems to investigate the spatiotemporal regulation of molecular pathways, they often carry adaptations that alter their mitochondrial quality control mechanisms. Whether the kinetics and regulation of mitophagy differ substantially between HeLa cells and primary neurons is an exciting future direction for study.
Figure 4.1 Mitophagy receptors are dynamically recruited to damaged mitochondria in response to PINK1- and PARK2-induced ubiquitination, leading to sequestration of the organelle by an LC3-positive phagophore. (1) An individual mitochondrion is acutely damaged. (2) The damaged mitochondrion is ubiquitinated through the action of Parkinson disease-linked proteins PINK1 and PARK2. (3) Within 15 min of PARK2-dependent ubiquitination, OPTN and its upstream kinase TBK1 are corecruited to the damaged mitochondrion. Simultaneously, autophagy receptors CALCOCO2 and TAX1BP1 translocate to the mitochondrial outer membrane. (4) Upon TBK1 phosphorylation, OPTN, associates with LC3, inducing autophagic engulfment of the damaged organelle. In the absence of TBK1 activity, CALCOCO2 can promote mitophagy. (5) Within 45 min of the initial injury, the damaged mitochondrion is sequestered within an autophagosome and functionally separated from the cytosol. (6) Over several hours, the mitochondrion is degraded within an autolysosome. Whereas Parkinson disease-linked mutations in PARK2 interfere with ubiquitination of damaged mitochondria, ALS-linked mutations in TBK1 and OPTN interfere with the subsequent recruitment of autophagy receptors and LC3-positive membranes.
CHAPTER 5: Dynamic actin cycling through mitochondrial subpopulations locally regulates the fission-fusion balance within mitochondrial networks


Supplementary movies can be accessed at: https://www.nature.com/articles/ncomms12886#supplementary-information

5.1 Abstract
Mitochondria form interconnected networks that dynamically remodel in response to cellular needs. Using live-cell imaging, we investigated the role of the actin cytoskeleton in regulating mitochondrial fission and fusion. We identified an unexpected cycling of actin filaments onto and off of subsets of cellular mitochondria. The association of actin filaments with mitochondrial subpopulations is transient; actin quickly disassembles then reassembles around a distinct subpopulation, efficiently cycling through all cellular mitochondria within 14 min. The focal assembly of actin induces local, Drp1-dependent fragmentation of the mitochondrial network. Upon actin disassembly, fragmented mitochondria undergo rapid fusion, leading to regional recovery of the tubular mitochondrial network. Cycling requires dynamic actin polymerization, and is blocked by inhibitors of both Arp2/3 and formins. We propose this cyclic assembly of actin onto mitochondria modulates the fission/fusion balance, promotes network remodeling and content mixing, and thus may serve as an essential mechanism regulating mitochondrial network homeostasis.
5.2 Introduction

Mitochondria are dynamic organelles that undergo fission and fusion to segregate their content of DNA, facilitate transfer of mitochondrial proteins, and enable mitophagic clearance of damaged organelles (Mishra and Chan 2014; Friedman and Nunnari 2014; Youle and van der Bliek 2012). The steady state balance between mitochondrial fission and fusion is a key determinant of overall mitochondrial network structure, and, by extension, cellular bioenergetics (Chen and Chan 2010). The processes of mitochondrial fission and fusion are regulated by a collection of large GTPases. Mitofusin 1 and Mitofusin 2 control fusion of the mitochondrial outer membrane (Santel and Fuller 2001), while Opa1 coordinates inner membrane fusion (Cipolat et al. 2004). In contrast, Drp1, through association with specific mitochondria-localized receptors, drives fission of both inner and outer mitochondrial membranes (Labrousse et al. 1999; Losón et al. 2013).

Prior to Drp1 recruitment, the endoplasmic reticulum marks prospective sites of mitochondrial fission (Friedman et al. 2011). ER tubules twist around mitochondria, inducing a pre-constriction event that decreases the mitochondrial cross-sectional diameter and allows for Drp1 assembly at the site of fission. The force necessary to drive this mitochondrial constriction is provided by actin polymerization by the ER-associated formin INF2 and the mitochondria-anchored formin-activating protein Spire1C (Korobova et al. 2013, 2014; Manor et al. 2015). Consistent with a key role for actin in the initial step of mitochondrial fission, actin depolymerization, or depletion of crucial actin-polymerizing proteins, result in increased mitochondrial length (Korobova et al. 2013; Li et al. 2015). Additionally, actin was shown to directly bind to and activate Drp1 (Ji et al. 2015), and is robustly recruited to fragmenting mitochondria after treatment with chemical ionophores (Li et al. 2015; Ji et al. 2015).
While the contribution of actin to mitochondrial fission has been examined at the level of the individual organelle, much less is known about how the actin cytoskeleton regulates the mitochondrial fission/fusion balance on a cell-wide level. To investigate this question, we used live-cell imaging to examine the dynamic interactions of actin with mitochondria. We made the unexpected observation that filamentous actin cyclically associates with distinct mitochondrial subpopulations in an Arp2/3- and formin-dependent manner to regulate mitochondrial networks within the cell. Actin dynamically assembles onto the outer membranes of a subpopulation of healthy, elongated mitochondria to promote fission and to inhibit fusion. Following mitochondrial fission, actin disassembles from the fragmented mitochondria, which rapidly re-fuse and reintegrate into the mitochondrial network. Actin subsequently assembles around a distinct, often neighboring mitochondrial subpopulation, and the process repeats. Over 14 minutes, actin cycles through all mitochondrial subpopulations, locally enhancing mitochondrial dynamics, which facilitates network remodeling and content mixing. Our study thus highlights novel actin dynamics during mitochondrial fission/fusion and identifies actin cycling as a homeostatic regulator of cellular mitochondrial morphology.

5.3 Results

5.3.1 Actin is associated with mitochondrial subpopulations

To explore the dynamics of actin assembly on mitochondria, we imaged live HeLa cells expressing LifeAct-GFP, a marker that preferentially labels filamentous actin (F-actin) (Riedl et al. 2008), and Mito-DsRed2, a mitochondrial matrix marker. As expected, the
The majority of actin filaments localized to the cell periphery and did not associate with mitochondria (Fig. 5.1a). However, we were also able to observe F-actin localization to subpopulations of mitochondria (Fig. 5.1a, Box 1). Using confocal microscopy, we identified clear LifeAct-GFP rings surrounding individual mitochondria (Fig. 5.1b,c), demonstrating robust recruitment of actin to the outer membrane of these organelles. Three-dimensional renderings of confocal z-stacks revealed F-actin cages entirely surrounding subpopulations of cellular mitochondria (Fig. 5.1d).

At any point in time, we found that LifeAct was recruited to only a subset (on average 23%) of the total mitochondrial pool (Fig. 5.1e). To query the functional consequence of this cytoskeletal association, we compared the size and morphology of actin-positive and actin-negative mitochondria within cells. We found that mitochondria with associated actin were significantly shorter, smaller, and less tubular (more circular) than mitochondria without associated actin (Fig. 5.1f,g,h). In parallel experiments in untransfected fixed cells, we observed localization of endogenous F-actin (visualized using Phalloidin) to 22% of TOM20-positive mitochondria (Fig. 5.1i,j,k; Fig. 5.1a). Consistent with our live cell data, we found that Phalloidin-positive mitochondria were comparatively more fragmented than those not associated with actin (Fig. 5.1b,c,d). In fixed cells, visualization of F-actin recruitment to mitochondria is most apparent in single confocal slices, but is also clearly visible in maximum intensity projections of 4 µm deep confocal stacks (Fig. 5.1e).

5.3.2 Actin cycles through mitochondrial subpopulations

Over time, we found that actin filaments did not remain stably associated with the same subset of mitochondria. Instead, we observed actin cycling through different mitochondrial
subpopulations. Imaging a live cell expressing LifeAct-GFP and Mito-DsRed2 over time shows the cycling of actin filaments from one subpopulation to another (Fig. 5.2a, b, c; Fig 5.S2; Supplementary Movies 1-4). The assembly and disassembly of actin filaments around specific mitochondrial subpopulations is most clearly seen in the enlarged images of specific regions of the cell (Fig. 5.2c), and the corresponding quantitation of the overall fluorescence intensity of LifeAct-GFP within these boxed regions (Fig. 5.2d). Actin remained associated with each subpopulation of mitochondria for approximately 3-5 min before depolymerizing (Fig. 5.2e). Upon depolymerization, actin subsequently repolymerized around a distinct, usually adjacent, subpopulation of mitochondria (note the clockwise movement of the arrows in the time series shown in Fig. 5.2a and Supplementary Movie 1), resulting in the robust cycling of actin through the entire mitochondrial network (see model in Fig. 5.2b).

To confirm our initial observations of actin dynamics made using LifeAct-GFP, we performed similar imaging experiments using either GFP-actin (Fig.5.S3; Supplementary Movie 5) or F-tractin-GFP (Belin et al. 2014) (Supplementary Movie 6) to visualize actin filaments. With all three probes, we noted very similar dynamics: actin transiently assembled around a subpopulation of mitochondria in the cell and subsequently cycled through distinct mitochondrial subpopulations (Fig. 5.S3; Supplementary Movies 1-6).

Given the persistent unidirectional cycling seen in some cells (Fig. 5.2a), we examined the directionality of cycling in the cell population. We found some cells exhibited persistent clockwise or counter-clockwise cycling, although most commonly we observed a more stochastic result in which the depolymerization of actin filaments from one mitochondrial population was equally likely to be followed by repolymerization around adjacent
mitochondria located to either side of the initial subpopulation. We also noted occasional apparent jumps across the cell to nonadjacent populations of mitochondria; however analysis of time-lapse movies composed of z-stack max projections revealed that the observed actin jumping was in fact actin cycling onto intervening adjacent subpopulations of mitochondria either above or below the nucleus that were outside of the initial confocal plane. The cumulative sampling of mitochondrial subpopulations in a clockwise fashion within a single cell is shown in the color-coded max projection of the distribution of F-actin through time (Supplementary Fig. 5.2c). The average rate of actin cycling through the cell’s total mitochondrial population is 14.0±0.8 min per cycle (mean±SEM; n=16 cells), with a stepwise migration event every 3.7±0.3 min (mean±SEM; n=16 cells).

Next we asked whether the phenomenon of mitochondrial actin cycling was restricted to HeLa cells, or could be seen in other cell types. We imaged LifeAct-GFP and Mito-DsRed2 in Cos-7 cells (Fig. 5.5, Supplementary Movie 7) and noted very similar dynamics of actin assembly and disassembly around mitochondrial subpopulations within a cell, demonstrating that this process is not unique to HeLa cells. We also queried whether similar actin dynamics could be observed in primary cells by imaging actin and mitochondria over time in normal human epidermal keratinocytes (Fig. 5.5, Supplementary Movie 8). Again, we saw the periodic assembly and disassembly of actin filaments around mitochondrial subpopulations, indicating that this phenomenon is not related to long-term cell culture or transformation.
5.3.3 *Actin cycling is not regulated by membrane potential or ROS*

Whole-cell depolarization of mitochondria induced by the mitochondrial uncoupler carbonyl-cyanide \( m \)-chlorophenyl-hydrazone (CCCP) has been reported to induce fission preceded by the rapid assembly of actin around mitochondria ([Li et al. 2015](#)). Consistently, we observed that within 2 minutes of CCCP addition, actin robustly assembled around the majority of mitochondria (Fig. 5.3a), effectively inhibiting further cycling of actin through mitochondrial subpopulations. CCCP-induced actin assembly on mitochondria was followed by robust mitochondrial fragmentation (Fig. 5.3a).

These observations led us to ask whether the dynamic assembly of actin on specific mitochondrial subpopulations might occur in response to fluctuations in membrane potential. First, we compared actin localization to the intensity of TMRE (tetramethylrhodamine ethyl ester), a fluorescent dye that accumulates in the mitochondrial matrix of healthy, polarized mitochondria but does not remain sequestered in the matrix of depolarized mitochondria. We found that mitochondria were uniformly polarized within each HeLa cell, as has been observed in neurons ([Verburg and Hollenbeck 2008](#)). We found no difference in the intensity of TMRE staining between the smaller, more circular mitochondria that were positive for actin and the longer, more tubular mitochondria that showed no actin association (Fig. 5.3b,c); further, we observed no cyclic alterations in TMRE intensity within a given cell over time that would correlate with the cyclic changes in actin ongoing in the cells. Thus, the association of actin filaments with subpopulations of cellular mitochondria is not correlated with fluctuations in their membrane potential.
Next, we asked if actin cycling onto specific mitochondrial subpopulations occurred in response to production of reactive oxygen species (ROS). We induced localized ROS production within the mitochondrial matrix by expressing the mitochondrially-targeted construct mito-KillerRed (Bulina et al. 2006) and then illuminating cells with 561-nm light. Whole cell illumination effectively bleached the KillerRed fluorescence, indicating activation of ROS production, but had no effect on the dynamic cycling of actin assembly/disassembly through mitochondrial subpopulations in time lapse imaging for up to 30 min (Fig. 5.S6).

Instead, the clearest predictor of actin cycling onto a mitochondrial subpopulation was mitochondrial size. We observed that actin was recruited to mitochondria with an average area of 2.24±0.13 μm² (mean±SEM), significantly larger than the average size of all cellular mitochondria (1.77±0.05 μm², mean±SEM, p<0.001; Fig. 5.3d).

5.3.4 Actin cycling promotes localized mitochondrial fission
As the dynamics of mitochondrial fission and fusion play an important role in maintaining mitochondrial homeostasis, we investigated a possible role for actin cycling in regulating mitochondrial morphology. Based on our observations that actin preferentially cycles onto elongated mitochondria (Fig. 5.3d) and that actin-positive mitochondria are more fragmented (Fig. 5.1f-h; Fig. 5.S1a-d), we hypothesized that actin recruitment promotes rapid mitochondrial fission. To investigate whether actin recruitment precedes and possibly promotes mitochondrial fission events, we closely examined subregions of the cellular mitochondrial network before, during, and after actin recruitment.
Prior to actin recruitment, mitochondria were typically long and tubular (Fig. 5.4a). Following actin polymerization, mitochondria became constricted (note specific sites indicated by yellow circles in Fig. 5.4a) and then fragmented into smaller pieces. Thus, actin recruitment to each mitochondrial subpopulation resulted in the localized fragmentation of mitochondrial networks (Supplementary Movie 9). In contrast, mitochondria that had not recruited actin within the same timeframe maintained their characteristic tubular and interconnected morphology.

We directly compared the kinetics of actin recruitment to changes in mitochondrial size (area and length) within specific regions of the cell, and found that these factors were inversely correlated (Fig. 5.4b,c). As actin intensity increased, the average individual mitochondrial size significantly decreased over a time scale of 4 min. Next, we examined the effects of localized actin assembly on the complexity of the mitochondria network. Over the same time scale and within the same localized region of the cell, we found that the increase in actin intensity was correlated with a decrease in the connectedness of the mitochondrial network, assessed as the number of branches per mitochondrion (Fig. 5.4d) and the number of junctions per mitochondrion (Fig. 5.4e; see Methods for details on the segmentation analysis used to assess these parameters).

We next assessed the frequency of mitochondrial fission in relation to actin association. The increased actin intensity indicative of dynamic actin assembly around a subpopulation of cellular mitochondria was closely correlated with a doubling in the number of mitochondria undergoing fission within this mitochondrial subpopulation (from 12.5±1.8 to 22.8 ± 2.7; Fig. 5.4f). To demonstrate that these changes were due to remodeling of the resident mitochondrial population rather than movement of mitochondria into or out of the
region of interest (ROI), we calculated the mitochondrial percent occupancy for each ROI, and found no significant change over time (Fig. 5.4g). Similarly, we tracked actin-associated mitochondrial fragmentation in z-stack projections (Fig. 5.S7), confirming that the observed fission is not due to the rotation or translocation of mitochondria in the z-axis.

Finally, to confirm that our actin markers were not contributing to the dynamic mitochondrial reorganization we observed upon actin recruitment, we performed live cell imaging of cells expressing only Mito-DsRed2. Even in the absence of actin markers, we observed distinct subregions of the mitochondrial network undergoing fragmentation concurrent with continued elongation of the mitochondrial network throughout the rest of the cell (Fig. 5.S8). Thus, our live imaging studies demonstrate that actin polymerization around mitochondrial subpopulations leads to a local enhancement of the frequency of fission, resulting in localized mitochondrial fragmentation and decreased interconnectivity.

5.3.5 Actin polymerization inhibits mitochondrial fusion

Assembly of actin around mitochondrial subpopulations clearly enhances fission, but we wondered whether this assembly might also inhibit fusion. Specifically, the formation of F-actin networks around mitochondria might decrease organelle motility and thus serve as a barrier to fusion with neighboring mitochondria. We found that, indeed, mitochondrial motility was significantly decreased when actin was assembled on mitochondria as compared to the motility of mitochondria without colocalized actin: we found the mean displacements for actin-positive and actin-negative mitochondria were 0.46±0.06 μm per sec and 1.03±0.12 μm per sec respectively (mean±SEM, p<0.001).
Next we asked whether actin disassembly was accompanied by an increase in the frequency of mitochondrial fusion. We focused on mitochondrial subpopulations enriched for actin, and assayed for effects on mitochondrial morphology as the associated actin filaments disassembled (Fig. 5.5a). Quantitative analysis demonstrates that the decline in the fluorescence intensity of LifeAct-GFP over a four-minute time course of actin disassembly is correlated with increased mitochondrial area and length (Fig. 5.5b,c; Supplementary Movie 10). The interconnectedness of the mitochondrial network also increased as actin disassembled, as we noted significant increases in both the number of branches per mitochondrion and the number of junctions per mitochondrion (Fig. 5.5d,e). Finally, there was a decrease in the total number of individual mitochondria (Fig. 5.5f), but no change in the percent occupancy within the examined ROI (Fig. 5.5g), consistent with an enhancement of fusion once actin disassembles from the given mitochondrial subpopulation. Tracking individual organelles, we observed that daughter mitochondria from fission events frequently moved in opposite directions before fusing with distinct, neighboring mitochondria, thus promoting content mixing and network remodeling (Fig. 5.5S9).

5.3.6 Actin cycling requires Arp2/3 and formins

The dynamics of actin cycling observed in live cells strongly suggest that polymerization of new, organelle-associated actin filaments is required. Therefore, this process should be inhibited by treatment with the actin-depolymerizing drug, Latrunculin B. Prior to treatment, we noted robust actin recruitment to a subset of mitochondria (Fig. 5.6a, left panel). After 20 min of Latrunculin B treatment, filamentous actin was not observed in association with any mitochondrial population in the cell (Fig. 5.6a,b).
Next we asked whether the assembly of actin filaments onto mitochondria was nucleated by either Arp2/3 or formins. Following a 1 h treatment of HeLa cells with the Arp2/3 inhibitor CK-666, actin cycling on mitochondrial subpopulations was completely abolished, and actin filaments were no longer associated with mitochondria (Fig. 5.6b). As recent work has implicated the formin INF2 and the formin-binding protein Spire 1C in mitochondrial fission, we also tested whether formin activity is required for dynamic actin cycling. We treated cells with the small molecule formin inhibitor SMIFH2 for 1 h. Again, we noted the complete inhibition of dynamic actin cycling, and the loss of actin association with mitochondria (Fig. 5.6b). Thus, both Arp2/3 and formins contribute to the dynamic formation of the mitochondria-associated actin filaments observed in our live cell assays, and inhibition of either nucleation mechanism is sufficient to block mitochondrial-associated actin cycling.

5.3.7 Fission of actin-positive mitochondria requires Drp1

Actin recruitment to mitochondria leads to enhanced fission, as shown above. This led us to ask whether the observed fission of actin-positive mitochondria is dependent on the canonical fission machinery. We first investigated the localization of Drp1, the dynamin-related GTPase that regulates mitochondrial fission, during actin cycling. In HeLa cells transfected with GFP-Drp1 we did not observe preferential recruitment of Drp1 to mitochondria positive for actin [Drp1 puncta on actin-positive mitochondria 1.21 ± 0.16 puncta per µm, Drp1 puncta on actin-negative mitochondria 1.23 ± 0.20 puncta per µm (mean ± SEM) p = .93]. In fixed cells as well, we observed comparable patterns of Drp1 localization.
staining on both Phalloidin-positive and Phalloidin-negative mitochondria, indicating that endogenous Drp1 is not preferentially recruited to actin-positive mitochondria.

Next, we asked whether Drp1 activity is necessary for actin-induced fission. We transfected cells with Drp1-K38A, a dominant negative mutant that has been shown to block mitochondrial fission (Smirnova et al. 1998). Consistent with previous reports, we observed robust mitochondrial hyperfusion in cells expressing Drp1-K38A. In cells transfected with Mito-DsRed2-, LifeAct-GFP, and Drp1-K38A, we identified actin cycling onto and off of subpopulations of elongated, interconnected mitochondria at a rate consistent with that observed in control cells (Fig 5.6c-e). However, actin recruitment did not induce remodeling of the mitochondrial network in cells expressing Drp1-K38A, in contrast to the robust remodeling observed in control cells (Fig 5.6c,d; See Fig. 5.S7 for 3D rendering). Similarly, in cells treated with the Drp1 inhibitor Mdivi-1 (Cassidy-Stone et al. 2008), actin cycling onto mitochondria was unaffected, but localized actin assembly did not promote robust mitochondrial fission (Fig. 5.S10). Quantitative analysis of z-stack projections indicate a significant increase in mitochondrial fission upon actin recruitment to mitochondria in cells transfected with an empty vector control, but only a modest change in mitochondrial number upon actin recruitment to mitochondria in Drp1-K38A expressing cells (Fig 5.6f). Similarly, we identified a 63% decrease in mitochondrial length upon actin recruitment in control cells, and only a 25% decrease in cells transfected with Drp1-K38A (Fig 5.6g). Therefore, actin cycles through mitochondrial subpopulations independently of Drp1, but Drp1 is required for efficient fragmentation and remodeling of the mitochondrial network following localized actin assembly.
5.3.8 Actin assembles at ER-mitochondria contacts

Recent work has identified key roles for ER-mitochondrial contact sites in lipid exchange, calcium signaling, as well as the initiation of mitochondrial fission (English and Voeltz 2013; Friedman et al. 2011). Thus we investigated whether actin preferentially cycles onto these inter-organelle junctions. Prior to actin recruitment, we frequently observed the ER in the vicinity of mitochondria (Fig. 5.7a). As actin polymerized on elongated mitochondria, we identified uniform actin recruitment to the outer mitochondrial membrane with specific enrichment of actin at sites of ER-mitochondria overlap (Fig 5.7a,b). Line-scan analysis indicated close apposition of actin with ER at sites of mitochondrial constriction prior to fission (Fig 5.7b,c). In 3D renderings of these ER-mitochondria contact sites, we see actin specifically enriched at points of mitochondrial constriction, where we observe the initiation of a fission event (Fig 5.7d). Thus, dynamic actin cycling may facilitate mitochondrial fission through the induction of ER-dependent mitochondrial constriction. This interpretation is consistent with recent work demonstrating a role for the mitochondrially-localized Spire 1C and ER-localized INF2 in the formation of mitochondrial constrictions (Korobova et al. 2013; Manor et al. 2015).

5.3.9 Effects of actin cycling on the mitochondrial network

Our data fit a model in which the active polymerization of actin on a mitochondrial subpopulation promotes fission, while actin disassembly allows fusion, locally restoring the integrity of the mitochondrial network (Fig. 5.8a). To quantitatively test this model, we directly compared mitochondrial length, number of branches and overall mitochondrial number in subcellular regions of comparable size: (1) before and after actin polymerization, (2) before and after actin depolymerization, and (3) in cellular regions in...
which no actin cycling was observed over a four-minute time course. As shown in Fig. 5.8b,c, active polymerization of actin led to a significant decrease in mitochondrial length within 4 min, with a corresponding increase in mitochondrial number. In contrast, actin depolymerization was followed by an increase in mitochondrial length and a decrease in mitochondrial number over a similar 4 min time course. Of note, in the absence of associated actin filaments, we noted a steady growth in mitochondrial length over the same 4 min window, and a small decrease in the number of mitochondria, suggesting that in the absence of external factors such as actin dynamics, fusion may dominate over fission.

To assess the effects of actin assembly/disassembly on the interconnectedness of the mitochondrial network, we measured the average number of branches per mitochondrion over time (Fig. 5.8d). Again, actin polymerization led to a significant fragmentation of the network, which was reversed upon actin depolymerization. As with mitochondrial length, we saw that in the absence of associated actin filaments, there is a tendency for the mitochondrial network to increase in complexity over time.

5.4 Discussion

In this study, we used live cell imaging to examine actin dynamics during mitochondrial fission and fusion. We propose that cyclic actin assembly/disassembly on mitochondrial subpopulations acts to regulate steady state mitochondrial morphology. Actin cycles through mitochondrial subpopulations in the cell, assembling on the outer membrane of elongated mitochondria to promote fission events and transiently inhibit mitochondrial fusion and motility. Subsequent disassembly of actin facilitates reconfiguration of the
mitochondrial network and mixing of mitochondrial contents. Cycles of actin assembly and disassembly survey and regulate the morphology of the total mitochondrial population within the cell in 14 min. In contrast to the chiral symmetry that the actin cytoskeleton can adopt in developing fibroblasts (Tee et al. 2015), we found that actin showed no directional bias in its recruitment to mitochondrial subpopulations. Further, we found no evidence for fluctuations in mitochondria membrane potential or reactive oxygen species production that might locally promote actin assembly around mitochondrial subpopulations.

At any point in time, actin filaments are associated with ~20% of cellular mitochondria. This association is transient, as actin remains assembled on mitochondrial subpopulations for 3-5 min. During this period, individual organelles exhibit enhanced fission and suppressed fusion, leading to localized fragmentation of the mitochondrial network. Consistent with previous reports (Friedman et al. 2011; Manor et al. 2015), we frequently observe fission events in the vicinity of mitochondria-ER contacts.

Fragmentation of actin-positive mitochondria is dependent on Drp1 activity, as inhibition of Drp1 by either treatment with the inhibitor Mdivi-1 or expression of a dominant negative Drp1-K38A blocks the local remodeling of actin-positive mitochondria. Of note, inhibition of Drp1 has no effect on the rate of actin cycling through the mitochondrial network, indicating that actin assembly and disassembly from mitochondrial subpopulations is not dependent on successful mitochondrial fission. Using both fixed and live cell techniques, we observed no differences in the localization or density of Drp1 puncta between actin-negative and actin-positive mitochondria. However, Drp1 puncta on actin-positive mitochondria may be differentially primed for fission through post-translational modifications (Harder et al. 2004; Chang and Blackstone 2010), association with outer-
membrane fission receptors such as Mff or Fis1 (Losón et al. 2013), or even simply through binding to actin itself (Ji et al. 2015).

Actin assembly on mitochondria results in a ~50% reduction in mitochondrial motility. This reduction in motility may contribute to the inhibition of mitochondrial fusion during local network remodeling. Specifically, decreased mitochondrial velocity reduces the probability of inter-mitochondrial contact events, which are necessary for successful fission (Cagalinec et al. 2013). Additionally, the formation of an actin cage around mitochondria may obstruct the association of mitofusin proteins between adjacent mitochondria, further potentiating the fusion block. Upon actin disassembly from a mitochondrial subpopulation, the organelles rapidly undergo fusion to mix their contents and restore a tubular mitochondrial network.

We found that F-actin recruitment to mitochondria is dependent on both Arp2/3 and formins. Curiously, inhibition of either actin-filament nucleator entirely abolished actin cycling through mitochondrial subpopulations, suggesting that Arp2/3 and formin family proteins work together to facilitate actin polymerization on the mitochondrial outer membrane. Work in budding yeast has identified Arp2/3-dependent actin clouds around fragmenting mitochondria (Boldogh et al. 2001). Additionally, Arp2/3 has been identified on the mitochondrial outer membrane of mammalian cells, where it regulates mitochondrial morphology (Li et al. 2015). Recently, the formin-binding protein Spire1C was identified on the mitochondria outer membrane, where it works in close association with the ER-localized formin INF2 to regulate mitochondrial length (Manor et al. 2015). Actin depolymerization by Latrunculin B, as well as depletion of Arp2/3 or certain formins results in robust mitochondrial elongation (Li et al. 2015; Korobova et al. 2013; Manor et al. 2015).
Moving forward, it will be interesting to examine the signaling pathways responsible for activation of actin nucleating proteins on elongated mitochondria, leading to localized filament assembly, as well as to investigate actin filament organization at higher resolutions.

We propose that actin cycling through mitochondrial subpopulations serves as a surveillance method to identify and fragment regions of elongated mitochondria. This hypothesis is consistent with previous observations indicating that elongated mitochondria are more likely to undergo fission (Cagalinec et al. 2013). Regional regulation of mitochondrial dynamics may confer a number of advantages in the maintenance of mitochondrial networks. First, localized mitochondrial network remodeling could be important for mitochondrial biogenesis and the maintenance and distribution of mtDNA nucleoids (Parone et al. 2008; Legros et al. 2004). Second, mitochondrial network remodeling may facilitate efficient transport of organelles through the cytoplasm, as previous work from our lab has demonstrated that interactions between organelles can restrict organelle trafficking (Zajac et al. 2013). An intricately connected mitochondrial network might have the same effect, such that local remodeling would be permissive for intracellular trafficking. Third, local remodeling of mitochondrial subpopulations allows for the fragmentation of individual, overgrown mitochondria without substantially altering the integrity of the mitochondrial network as a whole. As network-wide mitochondrial elongation has been linked to cellular senescence (Lee et al. 2007), actin cycling may be a highly important homeostatic function to offset premature senescence. Fourth, actin polymerization onto only one mitochondrial subpopulation at a time ensures that the cellular actin pool is not depleted, consistent with observations that most dynamic cellular actin is enriched at the cortex rather than in close association with mitochondria. Finally,
constitutive actin cycling may function as a quality control mechanism to isolate damaged or depolarized mitochondria, which are less able to re-fuse with other healthy organelles (Twig et al. 2008), for degradation by autophagy, again consistent with a homeostatic role for this mechanism.

Constitutive actin cycling may be efficiently tuned to maintain mean mitochondrial size, complexity, and number over each 14-minute cycle, while also maintaining heterogeneity in the morphology of individual mitochondria. During mitosis, cells in prophase and metaphase were found to have both more fragmented mitochondria and actin around their mitochondria (Li et al. 2015). Thus, actin cycling may be upregulated in mitotic cells whose mitochondrial network must efficiently fragment in preparation for segregation into daughter cells (Taguchi et al. 2007; Zunino et al. 2009; Kashatus et al. 2011). Actin cycling may also regulate mitochondrial morphology in post-mitotic cells such as neurons, which require precise regulation of mitochondrial dynamics and quality control for their viability (Chen and Chan 2006; Maday and Holzbaur 2014).

In summary, we propose a model in which mitochondrial network morphology is not regulated by a dynamic equilibrium between fission and fusion at the level of the individual organelle, but rather at a network level by the actin cytoskeleton. Mitochondria within the cell constantly grow and fuse, generating an increasingly branched and interconnected network. This steady growth is counterbalanced by intervals of fast and spatially restricted actin-dependent fission events, primarily at ER-mitochondrial contact sites. While we focused primarily on the HeLa model system, we note similar cycles of actin assembly/disassembly regulating mitochondrial fission and fusion in another cell line as well as in primary human cells, suggesting that actin-dependent cyclic mitochondrial
fission may be a conserved homeostatic mechanism that functions to regulate the cellular mitochondrial network.

5.5 Materials and Methods

5.5.1 Reagents

Constructs used include: EGFP-actin (Clontech), LifeAct-EGFP, F-tractin-EGFP (Addgene), mCherry-LifeAct (Dominguez lab, University of Pennsylvania), Mito-DsRed2 (gift from T. Schwarz, Harvard Medical School, Boston, MA) recloned into pSBFP2-C1 (Addgene) and SNAP-Tag (NEB), pKillerRed-dMito (Evrogen), Drp1 (Addgene) recloned into pEGFP (Clontech), Drp1-K38A (Addgene), DsRed2-ER (gift from A. Akhmanova, Utrecht University, Utrecht, Netherlands).

5.5.2 Cell culture and transfections

HeLa-M cells and Cos-7 cells were cultured in DMEM medium (10-027-CV, Corning) supplemented with 10% fetal bovine serum (vol/vol) and 1% glutamax and maintained at 37°C in a 5% CO₂ incubator. Normal human epidermal keratinocytes (NHEKs) were cultured from de-identified neonatal foreskin as previously described (Simpson et al. 2010). Cells were seeded in 35 mm glass bottom dishes and transfected ~24 h before imaging using FuGENE 6 (Promega). Cells were treated with 2 μM Latrunculin B for 30 min (BML-T110-0001, Enzo), 25 μM SMIFH2 for 1 h (S4826, Sigma), 84 μM CK-666 for 1 h (SML0006, Sigma), or 50 μM Mdivi-1 for 16 h (M0199, Sigma) prior to imaging. CCCP (Sigma-Aldrich) was given at 20 μM. Cells were incubated in 30nM TMRE in complete DMEM (T-669, Molecular Probes) for 15 min, and washed 2x in DMEM prior to live cell
imaging. Cells expressing Mito-SNAP were incubated with 2.5µM SNAP-cell 647-SiR (S9102S, NEB) for 30 min and washed 2x before imaging.

5.5.3 Live cell imaging and analysis

All images were acquired on a spinning-disk confocal (UltraVIEW VoX; PerkinElmer) on a Nikon Eclipse Ti microscope using an Apochromat 100× 1.49 NA oil immersion objective (Nikon) in a temperature-controlled chamber (37°C). Digital images were acquired with an EM charge-coupled device camera (C9100; Hamamatsu Photonics) using Volocity software (PerkinElmer) at 1 frame every 1-30 sec or 1 frame per min. Specifically, after the plates are mounted on the microscope, we selected the first observed cell with identifiable actin recruitment to mitochondria. The cell was imaged for no longer than 15-20 minutes with <10% laser power, and then immediately a second cell was imaged in a separate area of the plate. No more than 4 cells were imaged from a single plate. Cells were excluded from analysis if they displayed signs of phototoxicity such as blebbing or vacuolization. All experiments were performed on at least three independent occasions. Time-lapse maximum intensity projection movies were created by acquiring nine z-slices separated by 0.5 µm step size every 30 sec for 15-20 min. To induce ROS production, we photobleached a region or the entire HeLa cell expressing pKillerRed-dMito with a 561-nm laser at 100% for 100 iterations.

Images of mitochondria (both confocal slices and maximum intensity projections) were segmented using the interactive learning and segmentation toolkit (Ilastik 1.1.5) and analyzed by Fiji (NIH). Segmentation of TOM20 labeled mitochondria was carried out
using Volocity (PerkinElmer). Automated image analysis was manually verified by comparing the segmentation mask to the original image files. Measures of mitochondrial area, number, and fractional occupancy were generated using the “analyze particles” function in FIJI (NIH) with a minimum area of 0.25 µm$^2$. Measures of mitochondrial length, branches, and junctions were determined using the “skeletonize” and “analyze skeleton” plugins in FIJI (NIH). For analysis, ~100 µm$^2$ regions of interest were selected corresponding to the subpopulation of mitochondria targeted for actin recruitment.

For actin recruitment analyses in Fig. 5.4 and 5.8, time 0 is defined as 1 min prior to the first observable actin recruitment to >25% of mitochondria within the region of interest. For actin disassembly analyses in Fig. 5.5 and 5.8, time 4 is defined as 1 min subsequent to the disassembly of actin from >75% of mitochondria within the region of interest. For Fig. 7, segmentation of mitochondria from both control and Drp1-K38A cells was carried out on maximum intensity projections and mitochondrial length and number was normalized per cell. Line scans were generated using ImageJ (NIH) and normalized per fluorescent tag. Three-dimensional renderings were generated using Volocity (PerkinElmer). All images were assembled using Fiji (NIH) and Illustrator (Adobe). Statistics and graphing were performed using Prism (GraphPad) software. Comparisons of two data sets were performed using unpaired two-tailed Student’s t test. Comparisons for multiple data sets were performed using one-way ANOVA with Tukey’s post-hoc test.

5.5.4 **Immunofluorescence**

HeLa cells seeded on 35 mm glass bottom dishes were fixed in 3.7% paraformaldehyde at 37°C for 12 min, washed in PBS 3x, and permeabilized in 0.2% Triton X-100 in PBS for
no longer than 5 min at RT. Cells were again washed in PBS 3x, and blocked in 0.5% BSA in PBS for 10 min at room temperature. Cells were then incubated for 30 min with Phalloidin-488 (A12379, ThermoFisher), anti-Tom20 (sc-11415, Santa Cruz), and anti-DRP1 (ab56799, abcam) in PBS with 0.5% BSA. Cells were then washed in PBS 3x, and incubated with AlexaFluor goat-anti-rabbit-594 (150084, abcam), AlexaFluor goat-anti-mouse-647 (150115, abcam), and Hoechst dye. Cells were washed in PBS 3x and imaged.

5.5.5 Acknowledgements

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5.5.6 Author Contributions

A.S.M. co-designed the study, performed experiments, analyzed the data and wrote the manuscript; Y.C.W. co-designed the study, performed experiments, analyzed the data and wrote the manuscript; C.L.S. performed experiments and edited the manuscript, E.L.F.H. co-designed the study and wrote the manuscript.
5.6 Figures

5.6.1 Fig. 5.1.

Figure 5.1. F-actin localizes on the outer membrane of a mitochondrial subpopulation.

(a) Confocal microscopy image of F-actin (LifeAct-GFP) localization to a subset of Mito-DsRed2 labeled mitochondria in HeLa cells.  (b) Enlarged images showing fragmented, actin-positive mitochondria (Box 1) and elongated, tubular mitochondria which have not recruited actin (Box 2).  (c) Line scans indicating actin-positive mitochondria (top) and actin negative mitochondria (bottom).  (d) Three-dimensional renderings of z-stack images showing differential morphologies of actin-positive mitochondria (top) and actin-negative mitochondria (bottom) within the same cell.  (e) Percentage of actin-positive and actin-negative mitochondria per cell.  (f,g) Mitochondria that have recruited actin have decreased length and area.  (h) Actin-positive mitochondria are significantly more rounded than actin-negative mitochondria.  (i) Confocal image of Phalloidin-488 recruitment to TOM20 labeled mitochondria in a fixed HeLa cell.  (j) Enlarged images displaying Phalloidin recruitment to fragmented but not elongated mitochondria.  (k) Line scans showing colocalization of the TOM20 outer membrane marker with Phalloidin (top) and TOM20 without actin colocalization (bottom). Values represent means ± S.E.M.  ***p < 0.001, n.s. (not significant).  Scale bars (a,i), 10 µm; (b, d, j), 2.5 µm. Sample size (e), 19 cells; (f), 9 cells; (g), 10 cells; (h), 26 cells.  N≥3 independent experiments in all cases.
5.6.2 **Fig. 5.2.**

(a) Confocal time series showing F-actin (LifeAct-GFP) cycling through different mitochondrial subpopulations within the cell in a clockwise direction over 10.5 minutes. Large arrow indicates leading edge of actin-positive mitochondria; small arrow indicates trailing edge of actin-positive mitochondria. (b) Model of actin cycling through mitochondrial subpopulations over time corresponding to a. (c) Enlarged images of Boxes 1-3, showing temporally ordered actin polymerization/depolymerization on individual mitochondria (white arrows). (d) Normalized actin intensities within Boxes 1-3 over time. (e) Histogram of F-actin residence time on individual mitochondria during cycling. Values represent means ± S.E.M. *** p < 0.001. Scale Bars (a), 10 µm; (c), 2.5 µm. Sample size (e), n= 204 mitochondria from 22 cells; N≥3 independent experiments.
Figure 5.3. Actin cycles onto polarized, elongated mitochondria in healthy cells.

(a) Time series demonstrating stable recruitment of F-actin (LifeAct-GFP) to mitochondria (Mito-DsRed2) in response to depolarization by 20 µM CCCP. Prior to CCCP treatment, cycling actin can be visualized on a subpopulation of HeLa cell mitochondria (yellow arrow, top panel). Within 2.5 min of CCCP treatment, actin is stabilized on > 90% of all mitochondria (red arrows), inducing robust fission of all mitochondria by 15 min. (b-c) Untreated cells expressing LifeAct-GFP and Mito-BFP display no significant difference in TMRE intensity between actin-positive and actin-negative mitochondria. (d) Mitochondria targeted for actin assembly are significantly larger than average mitochondria within the cell. Values represent means ± S.E.M. ***p<0.001. Scale bars (a full size), 10 µm; (a zoom, b), 2.5 µm. Sample size (c), 50 mitochondria from 10 cells per condition; (d, whole network), 27 cells; (d, actin-targeted subpopulation), 26 cells. N≥3 independent experiments in all cases.
5.6.4 Fig. 5.4.

Figure 5.4. Actin cycling through a mitochondrial subpopulation induces mitochondrial fragmentation.

(a) Confocal time series of actin (LifeAct-GFP) cycling onto elongated, tubular mitochondria (Mito-DsRed2). Over 4 min, actin-positive mitochondria become increasingly fragmented. Yellow circles indicate sites of mitochondrial constriction. (b,c) Mitochondrial area and length decrease as actin intensity increases. (d,e) Actin recruitment affects mitochondrial shape complexity, resulting in fewer branches and junction points per mitochondrion. (f,g) Actin cycling onto mitochondria results in increased mitochondrial number, but no change in the overall mitochondrial percent occupancy within the fixed ROI, indicative of fission. Values represent mean ± S.E.M. Scale bars (a), 2.5 µm. Sample size (b-g), 6 cells; N=3 independent experiments.
Figure 5.5. Mitochondria undergo rapid growth upon actin disassembly.

(a) Confocal time series showing actin (LifeAct-GFP) depolymerization from a subpopulation of fragmented, rounded mitochondria (Mito-DsRed2). Upon actin disassembly, mitochondria undergo rapid growth and fusion. (b,c) Mitochondrial area and length increase as actin intensity decreases. (d,e) Actin depolymerization augments mitochondrial complexity, resulting in an increased number of mitochondrial branches and junctions. (f,g) Actin cycling off of mitochondria precedes fusion, but has little effect on total mitochondrial percent occupancy. Values represent mean ± S.E.M. Scale bars (a), 2.5 µm. Sample size (b-g), 9 cells; N≥3 independent experiments.
5.6.6 Fig. 5.6.

(a) Confocal images of F-actin (LifeAct-GFP) localized to a mitochondrial subpopulation (MitoDsRed2) during constitutive cycling (left). Actin recruitment to mitochondria is abolished upon 20 min treatment with the actin depolymerizing drug Latrunculin B (2 µM) (right). (b) Actin cycling is abolished in cells treated with 2 µM Latrunculin B (30 min), 84 µM CK-666 (1 h), or 25 µM SMIFH2 (1 h). (c) Expression of Drp1-K38A results in increased mitochondrial length and interconnectivity, but does not block actin cycling onto mitochondrial subpopulations. (d) In cells expressing an empty vector control, actin recruitment results in significantly decreased length, followed by recovery (for 3D rendering of mitochondria undergoing fragmentation, see Supplementary Fig. 7). (e) In cells expressing Drp1-K38A, actin recruitment does not significantly affect the length of targeted mitochondria. Values represent mean ± S.E.M. ***p < 0.001, **p < 0.01, n.s. = not significant. Scale bars (a full size) 10 µm; (c, d) 5 µm; (a, zoom) 2.5 µm. Sample size (b, DMSO), 9 cells; (b, Latrunculin), 91 cells; (b, CK-666), 101 cells; (b, SMIFH2), 24 cells; (e), 16 cells; (f,g control) 15 cells; (f,g, K38A), 14 cells; N≥3 independent experiments in all cases.
Figure 5.7. Actin assembles at ER-mitochondria contact sites.

(a) Confocal image of GFP-actin (red) recruitment to elongated, Mito-SNAP labeled mitochondria (blue). Actin assembly is enriched at sites of ER tubule (DsRed2-ER, green) overlap with mitochondria. Arrows indicate regions of Actin/ER colocalization at prospective sites of mitochondrial fission. (b) Enlarged image of Box B (+ 4 min), demonstrating colocalization of actin and ER at the site of mitochondrial fission. (c) Line-scan indicating overlapping peak actin and ER intensity at site of mitochondrial constriction in b. (d) Three-dimensional rendering of F-actin (LifeAct-GFP) and ER-tubules (DsRed2-ER) assembled on constricted mitochondrion (Mito-SNAP). Scale bars (a) 1µm, (b) 0.5 µm, (c) 0.75 µm per unit.
Figure 5.8. Actin cycling through mitochondrial subpopulations regulates steady-state mitochondrial morphology.

(a) Model: Actin polymerizes on a subpopulation of elongated mitochondria, inducing robust fragmentation. After ~3-5 min, actin then depolymerizes, allowing the fragmented mitochondria to refuse, and slowly increase in size and complexity. Actin-negative mitochondria slowly continue to grow until they become locally hyperfused, at which point actin is recruited and the cycle repeats. (b,c) Mitochondrial size and complexity decrease with actin recruitment, and recover upon subsequent actin depolymerization. (d) Actin polymerization induces rapid mitochondrial fission, while depolymerization promotes slow fusion. Values represent mean ± S.E.M. ***p < 0.001, **p < 0.01, *p < 0.05, n.s. (not significant). Sample size (b-d, actin polymerization), 26 cells; (b-d, actin depolymerization), 9 cells; (b-d, actin negative), 32 cells; N≥3 independent experiments in all cases.
5.6.9  **Fig S1.**

*Phalloidin localizes to fragmented mitochondria in fixed HeLa cells.*

(a) Percentage of Phalloidin-positive mitochondria per cell. (b-d) Phalloidin-positive mitochondria are shorter in length, smaller in area, and more circular than Phalloidin-negative mitochondria. (e) Maximum intensity projection (4 z-slices separated by 1 µm intervals) of cell displayed in figure 1i indicating that the decreased size of Phalloidin-positive mitochondria is not due to the plane of sectioning. Error bars represent mean ± S.E.M. ***p < 0.001. Scale bars (e full size) 10 µm; (e zoom) 5 µm. Sample size (a), 24 cells; (b-d), 31 cells; N≥3 independent experiments in all cases.
Fig. 5. S2. F-actin cycles through mitochondrial subpopulations.

(a) Confocal time series of F-actin (LifeAct-GFP) cycling through subpopulations of mitochondria (Mito-DsRed2).  (b) Enlarged images of Boxes 1-3. White arrows indicate actin-positive mitochondria.  (c) Pseudocolored maximum intensity projection of F-actin localization over 12.5 min.  (d) Normalized intensity of LifeAct-GFP in boxes 1-3 over time. Scale bars (a,c), 10 µm; (b), 2.5 µm
Fig. 5.S3. Actin cycles through mitochondrial subpopulation over time.

(a) Confocal time series of actin (GFP-actin) recruitment to distinct populations of mitochondria (Mito-DsRed2) over 15 min. (b) Enlarged images of boxes 1-4 demonstrating actin polymerization and depolymerization from individual mitochondria. (c) Model showing actin cycling in a persistent clockwise direction, sampling all mitochondria within the cell. (d) Normalized intensity of GFP-actin in boxes 1-4 over 15 min. Scale bars (a), 10 µm; (b), 2.5 µm.
Fig 5.S4 Actin cycles through mitochondrial subpopulations in Cos-7 cells.

(a) Confocal image of F-actin (LifeAct-GFP) recruitment to mitochondria (Mito-DsRed2) in Cos-7 cells. (b) Enlarged images of Boxes 1-2, indicating actin polymerization onto mitochondria in Box 1 and depolymerization from mitochondria in Box 2 over 15 min. (c) Normalized intensity of LifeAct-GFP in Boxes 1 and 2 over 14 min. Scale bars (a), 10 µm; (b), 2.5 µm.
Fig 5.5: Actin cycles through mitochondrial subpopulations in normal human epidermal keratinocytes

Confocal image of normal human epidermal keratinocyte (NHEK) expressing LifeAct-GFP and Mito-DsRed2 (left). F-actin cycles from mitochondria in Box 1 to Box 2 over 3.5 min. Between 3.5 and 5.5 min, actin cycles from mitochondria in Box 2 to Box 3. Scale bars (left) 10 µm; (right), 2.5 µm.
Increased ROS production does not disrupt actin cycling through mitochondrial subpopulations.

(a) Model: Illumination of mitochondrial-targeted Mito Killer Red by 561 nm laser light generates reactive oxygen species (ROS) within the mitochondrial matrix. (b) Confocal time series of HeLa cell expressing LifeAct-GFP, Mito-BFP, and Mito Killer Red. Activation of Mito Killer Red by photobleaching does not impede actin cycling over time. Yellow arrow indicates subpopulation of actin-positive mitochondria. Scale bars (b, full size), 10 μm, (b, zoom), 2.5 μm.
Fig 5.S7. Actin polymerization induces mitochondrial fission and local mitochondrial network remodeling.

3D-rendering of an individual mitochondrial subpopulation over 14 min. Mito-DsRed2 labeled mitochondria within the displayed subregion are initially elongated and interconnected. As F-actin cycles onto the elongated mitochondria (white arrows) the mitochondria undergo robust fission. Dashed circles indicate sites of mitochondrial fission. By 14 min, actin has cycled off of the mitochondria in the right-hand side of the window, and the fragmented mitochondria begin to fuse (yellow asterisks) and recover their tubular morphology. See Fig. 6c for enlarged max projection of right hand region. Scale bar 5 µm.
In the absence of actin markers, mitochondrial networks undergo regional growth simultaneous to localized fragmentation. 

(a) Confocal image of HeLa cell transfected with Mito-DsRed2. Box 1 indicates a subregion of the mitochondrial network that is more interconnected, while Box 2 indicates a subregion of the mitochondrial network that is more fragmented. (b) Mitochondria within Box 1 become increasingly fragmented over ten min, while mitochondria in Box 2 become increasingly interconnected. Scale bar (a) 10 µm; (b) 2.5 µm.
**Fig. 5.S9.** Fragmented mitochondria rapidly fuse after actin depolymerization.

Confocal time series indicating actin cycling onto and off of an individual mitochondrion (blue arrow). Over 4 min, actin polymerization promotes fission of the indicated mitochondrion (dashed circle, t=4 min), resulting in the production of two daughter mitochondria. Over the subsequent 4 min, one of the daughter mitochondria (left blue arrow) comes into close contact with a separate, adjacent mitochondrion (yellow arrow), eventually fusing (green arrow). Over the same time period, the other daughter mitochondrion remains fragmented (right blue arrow). Scale bar 1 µm.

**Fig. 5.S10.** Actin does not promote mitochondrial fragmentation upon Drp1 inhibition.

Actin cycling is preserved in HeLa cells pre-treated with the Drp1 inhibitor Mdivi-1 (50 µM for 16 h). However, actin polymerization on mitochondrial subpopulations does not promote robust mitochondrial fragmentation. Scale bar, 5 µm.
CHAPTER 6: CYCLING ACTIN CLOUDS ENSURE THE FIDELITY OF MITOCHONDRIA NETWORK INHERITANCE
6.1 Introduction

Throughout development and into adulthood, organisms must continue to generate new cells in order to grow and respond to injury. Each time a cell divides, it must not only coordinate the equal segregation of nuclear DNA, but also ensure the symmetric and random inheritance of mtDNA. Random partitioning of mtDNA between daughter cells, is critically important to prevent co-inheritance of mtDNA mutants, potentially leading to the clonal expansion of those mutants and genetic drift between daughter cell lineages.

To date, several mechanisms of mitochondrial inheritance have been proposed. In asymmetrically dividing yeast, mitochondria are transited along actin cables via the class V myosin, myo2, and sorted into either mother or bud (Altmann et al., 2008). This myosin-based mitochondrial motility can be supplemented by an Arp2/3-dependent actin cloud that assembles on mitochondria and promotes their rapid movement over short distances (Boldogh et al., 2001) or by tethering to the cortical endoplasmic reticulum (Swayne et al., 2011).

In more complex eukaryotes, mitochondrial motility in interphase cells is predominantly regulated by the microtubule cytoskeleton. Specifically, the adaptors RhoT1/2 and TRAK1/2 link mitochondria to the microtubule motors dynein and kinesin (Guo et al., 2005; van Spronsen et al., 2013). However, as cells enter mitosis, the microtubule network undergoes a rapid and dramatic rearrangement to form the mitotic spindle. During this period, mitochondria fragment and detach from their microtubule tracks, moving to the cell periphery where they remain until early anaphase (Chung et al., 2016; Kanfer et al., 2015). It has been proposed that mitochondria passively drift during this period and are
stochastically segregated at the completion of cytokinesis. To investigate this claim, we set out to observe mitochondria network dynamics in dividing mammalian cells.

**Mitochondria orientation shifts in mitosis**

Imaging high-speed mitochondrial dynamics during cell division poses several challenges to conventional optical sectioning microscopy techniques. In flat, interphase cells, we find that mitochondria are tightly associated with the microtubule cytoskeleton and predominantly oriented with their long axis parallel to the substratum (Fig 6.1A). As a result, motile mitochondria largely remain in the focal plane during time lapse movies. However, upon m-phase entry, mitochondria detach from their microtubule tracks and the cell increases in height by a factor of 2-3 (Fig 6.1A, Fig 6.S1A). No longer bound to microtubules, mitochondria shift such that in a majority of cases their long-axis is positioned perpendicular to the XY plane (Fig 6.1A-E, Fig S6.1B-G). Consequently, volumetric imaging is required to accurately assess mitochondrial morphology and dynamics.

In order to section through metaphase mitochondrial networks, cells must be exposed to prolonged laser irradiation, potentially resulting in phototoxicity and fluorophore bleaching. Further, the larger z stack size increases acquisition time, prohibitively limiting the temporal resolution of live cell movies. Finally, refractive index mismatch between a high-NA oil immersion objective lens and the aqueous sample results in spherical aberrations that further decrease signal to noise. To overcome these obstacles, we used a combination of spinning-disk confocal and lattice light-sheet microscopy, which enabled high-speed, high-resolution volumetric imaging of mitochondrial dynamics through cell division.
6.1.1 Mitochondria undergo robust, microtubule-independent motility in metaphase

In contrast to a passive-drift model, we observe robust mitochondrial motility in metaphase cells. Labeling mitochondria with either genetically encoded fluorophores (Mito-DsRed2) or cationic dyes (MitoTracker CMxROS and MitoTracker Deep Red), we saw that mitochondrial movements matched and often exceeded interphase mitochondrial motility. This observation was consistent across numerous cell lines, including HeLa, iPSC, HaCaT, HEK293T, MCF7, Cos7, A549, and Normal Human Epidermal Keratinocytes (NHEK) (Fig 6.S2A-D).

Multiple lines of evidence indicate that metaphase mitochondrial motility is not dependent on the microtubule cytoskeleton. First, high resolution iSIM and expansion microscopy images show that mitochondria are entirely excluded from the mitotic spindle and show only limited overlap with astral microtubules (Fig 6.S2E,F; Chung et al., 2016). Second, microtubule depolymerization by nocodazole treatment (25µM) largely stalled mitochondrial movements in interphase cells but did not decrease mitochondrial movements in metaphase cells (Fig 6.1F).

6.1.2 Actin clouds rapidly cycle through metaphase cells

Several investigations have found that the actin cytoskeleton can regulate short-range mitochondrial motility (Quintero, 2009). Additionally, actin has been implicated in mitochondrial inheritance in yeast and mammals (Boldogh et al., 2001; Rohn et al., 2014). Thus, we investigated whether actin could be driving the observed mitochondrial motility.
To investigate the role of the actin cytoskeleton in regulating mitochondria network inheritance, we generated stable HeLa lines expressing the F-actin marker Lifeact-EGFP as well as a mitochondria matrix targeted red fluorescent protein (Mito-DsRed2). We then used LLSM and spinning disk to track actin and mitochondria dynamics as these cells underwent mitosis (Fig 6.2A).

In metaphase cells, we observed a large three-dimensional cloud of actin filaments encompassing approximately 1/4 of all mitochondria (Fig 6.2b, Fig 6.S3A,B). This deep cytoplasmic actin structure is easily concealed by the dense cortical actin network but is readily apparent in images obtained with sufficient contrast.

Over time, we observed that the actin cloud travels through the cell as a unidirectional wave, propagating along mitochondria and completing a full revolution every 6 minutes (Fig 6.2C, Fig 6.S3C). Actin clouds move at a relatively uniform velocity (Fig 6.S3D) and display a bias for persistent, unidirectional motility (Fig 6.S3E). Three-dimensional time projection (Fig 6.2D) and autocorrelation analysis (Fig 6.2E) reveal the highly periodic nature of this waveform. We observe that actin clouds persist through all stages of mitosis (Fig 6.S3F), and preferentially associate with mitochondria localized to the cleavage furrow in telophase (Fig 6.S3G). Upon the completion of cytokinesis, the actin cloud is divided between daughter cells.

Previous work from our group identified a similar cloud of actin filaments in interphase mitochondria (Fig 6.S4A,B). This cloud moved at a rate of ~ µm/min, transiently driving mitochondrial fission (Fig 6.S4C-E; Moore et al., 2016). We find that metaphase actin
waves have a significantly higher frequency and velocity, propagating through mitochondria networks nearly three times faster than interphase actin clouds (Fig 6.2F,G). This increased velocity is due to shorter dwell time of actin on metaphase mitochondria and significantly faster kinetics of actin assembly and disassembly (Fig 6.2H-J).

We hypothesized that enhanced actin waves in metaphase cells may be due to changes in the geometry of rounded, mitotic cells. To investigate this, we transfected cells with Rap1Q63E, a dominant negative construct that inhibits mitotic rounding. Despite decreased rounding in metaphase, we still observed rapidly cycling actin clouds in metaphase. Next, we plated interphase HeLa cells on micropatterned substrates of various sizes and geometries. However, we found that constraining cell boundaries had no effect on actin cloud velocity (Fig 6.S4F-H). Together, these observations suggest that there is a chemical change that occurs at the G2/M transition that enhances actin cycling.

Of note, several groups have identified similar subcortical actin clouds in metaphase cells, proposing that they play roles in spindle orientation and cleavage furrow formation (Mitsushima et al., 2010; Fink et al., 2011; Kwon et al., 2010). Crucially, none of these investigations has documented the mitochondrial dependence that we observe.

6.1.3 Actin clouds require Arp2/3, N-WASP, and CDC42

In order to elucidate the mechanism of mitochondrial actin-cloud assembly, we screened several small molecule inhibitors of actin dynamics and queried their effect on mitochondrial actin cloud assembly (Fig 6.3A). We observed that inhibitors of Rho and Rac GTPases robustly altered cellular actin structures without eliminating or
downregulating mitochondrial actin assembly. Similarly, inhibition of Myosin II or its upstream kinase ROCK did not affect actin cloud size or velocity. However, inhibition of either CDC42 or its downstream effector N-WASP resulted in a significant decrease in the percentage of cells containing mitochondrial actin clouds (Fig 6.3A). Of note, we found that treatment of cells with either the Arp2/3 inhibitor CK-666 or the pan-formin inhibitor SMIFH2, robustly downregulated the percentage of cells with visible actin clouds (Fig 6.3A, Fig 6.S5A). Depletion of Arp3 by siRNA showed a similar inhibition of actin cycling (Fig 6.3B).

To further establish the actin binding proteins involved in mitochondrial actin clouds, we transfected stable Lifeact-mScarlet HeLa cells with various GFP-tagged actin binding proteins. We observed robust colocalization of cortactin, alpha-actinin, and filamin A with mitochondrial actin clouds (Fig 6.S5B-G). Filamin A localization to actin clouds was further confirmed by immunostaining (Fig 6.S5C). Depletion of filamin A or cortactin failed to eliminate metaphase actin clouds, suggesting that these ABPs are either not necessary for actin cloud propagation or can be functionally compensated for by other molecular players (data not shown).

6.1.4 Actin clouds require CDK1

Based on the enhanced cycling that we observed in metaphase cells, we hypothesized that the mitotic kinase CDK1 is required for actin cloud propagation. As CDK1 inhibitors block mitosis and promote mitotic exit, we could not evaluate the effect of these drugs on metaphase actin clouds. However, we found that two independent CDK1 inhibitors robustly blocked the formation of actin clouds in interphase cells (Fig 6.3C). Inhibition of
other kinases, including PKC and ERK did not affect actin assembly on mitochondria (Fig 6.3C).

6.1.5  Actin clouds drive rapid bursts of mitochondrial motility in metaphase cells

We hypothesized that the rapidly cycling actin cloud may be responsible for the microtubule-independent mitochondrial motility that we observed in metaphase cells. To investigate this hypothesis, we assessed total mitochondrial network motility using displacement index (DI) analysis (Quintero et al., 2009). Mitochondria were imaged at 2 seconds per frame for 5 minutes and the resulting time lapse movie was compressed into a single frame maximum intensity projection. We then divided the area of the mitochondria in the maximum projection by the mitochondrial area in frame 1 of the movie.

Cells transfected with a non-targeting control siRNA had a significantly higher DI (4.00±0.38 n=39 cells) than those treated with Arp3 siRNA (3.02±0.36 n=33 cells; p<0.001; Fig 6.3D,E,H). We observed a similar difference in DI between untreated cells and those incubated for 1h with the actin depolymerizing drug CytoD (Fig 6.3F,G,H). Of note, inhibition of actin cycling did not affect total mitochondrial area (untreated: 4.17±0.39, CytoD: 2.97±0.38, p<0.001; Fig 3I).

A major outcome of metaphase mitochondrial movements is the dynamic exploration of mitochondria through available cytoplasmic space. We found that over five minutes, mitochondria in control cells covered 70% of the cellular area, only failing to explore the interior of the cell containing the spindle. In contrast, mitochondria in Arp3 depleted cells only covered ~55% of cellular area (Fig 6.3K). This decrease was not due to changes in
overall cell shape, as control and Arp3 siRNA treated did not affect total cell area (Fig 6.3J).

Next, we acquired two-color movies of LifeAct-EGFP and Mito-DsRed2 in metaphase HeLa cells and compared the velocity of actin-positive and actin negative mitochondria. We observed that actin positive mitochondria moved at an average speed of 239 nm/sec (n=125 from 25 cells), more than twice as fast as actin negative mitochondria (109 nm/sec; n = 125 from 25 cells; Fig. 3L)

Bursts of actin assembly are typically associated with decreased organelle motility, likely through affecting cytoplasmic viscosity. Here, we instead find that F-actin assembly on mitochondria actually drives mitochondrial movement. To reconcile these points, we carefully examined high speed timelapse movies, and observed that mitochondria motility peaks during the initial ~30 seconds of actin polymerization on the mitochondrial membrane. During this time, actin only partly encircles the mitochondrial membrane and the organelle typically displaces in the opposing direction. Once the mitochondrion is fully encased by F-actin, the mitochondrial motility drops (Data not shown). Of note, Arp2/3 mediated actin assembly has previously been shown to drive motility of organelles and even invasive pathogens (Welch et al., 1997; Taunton et al., 200).

6.1.6 Actin based mitochondrial motility is constrained by the ER

We observed that the total displacement of actin-positive mitochondria was typically <5 µm, but mitochondria often moved out of the focal plane and could not be tracked. Additionally, we found that motile metaphase mitochondria often displayed highly curved
trajectories. Based on these observations, we reasoned that actin-mediated mitochondrial motility was constrained, potentially by interactions between mitochondria and the endoplasmic reticulum (ER).

In mitosis, the ER converts from a predominantly tube-like network to a series of vertical cisternae (Lu et al., 2009). Using multi-color confocal microscopy, we see that individual mitochondria are enclosed within ER cisternae in metaphase cells. Upon actin assembly, mitochondria rapidly displace, but their total motility is constrained by the local cisternal geometry (Fig 6.S6C). Depolymerization of astral microtubules by nocodazole treatment strikingly alters ER morphology (Smyth et al., 2015; Fig 6.S6D). In these cells, the ER appears to form a series of concentric, regularly spaced layers. Crucially, the ER membrane forms fewer cisternae, and thus poses fewer obstacles to mitochondrial motility. Consequently, actin positive mitochondria travel farther in metaphase arrested cells.

6.1.7 Actin based mitochondrial motility requires release from microtubules

Though we observe here that fast-moving actin clouds drive mitochondrial motility in metaphase cells, we previously found that slower moving interphase actin clouds drive mitochondrial fission (Moore et al., 2016). We hypothesized that this differential effect of actin assembly was due to mitochondria-microtubule interactions. In interphase cells, mitochondria are tightly associated with the microtubule cytoskeleton via molecular motors, including dynein and kinesin, and microtubule tethers, such as syntaphilin. Because mitochondria are bound to static microtubules the force of actin assembly may not be transduced into motility but instead result in membrane deformation and ultimately
fission. In contrast, metaphase mitochondria are untethered from their microtubule tracks, so the force of actin assembly can directly drive mitochondrial displacement. Indeed, when we depolymerized interphase microtubules, we found that actin clouds were able to promote mitochondrial movement, much like metaphase cells (Fig 6.S7A,B).

6.1.8 *Actin cycling ensures homogenous distribution of mitochondria through metaphase cells*

We observe that bursts of actin-mediated mitochondrial motility drive mitochondrial distribution. To visualize mitochondrial mixing, we transfected cells with a mitochondrial matrix targeted photoactivatable GFP (mito-paGFP). We then briefly irradiated a small region of the mitochondrial network with 405nm light in order to photoactivate mito-paGFP, and tracked the localization of those mitochondria over time by kymograph analysis (Fig 6.4a). We observed that photoactivated mitochondria displaced in the direction of actin cycling (Fig. 6.4a). Inhibition of actin cycling, but not other actin dependent phenomena, resulted in mitochondrial clumping and inhomogeneous distribution of organelles throughout the cytoplasm (Fig 6.4B,C, Fig 6.S8A).

6.1.9 *Actin cycling shuffles the spatial localization of mtDNA nucleoids*

As mtDNA are the basic functional unit of mitochondrial inheritance, we sought to examine the effect of actin cycling on mtDNA nucleoid distribution. We labeled cells with the mtDNA dye pico green, and incubated cells with either DMSO or CytoD. In DMSO treated cells, mtDNA nucleoids appeared to uniformly positioned throughout the cytoplasm (Fig 6.4D-F). In contrast, CytoD resulted in clumped nucleoids and large areas of cytoplasm without
mitochondria (Fig D, bottom row). Similar results were obtained using SNAP-TFAM to label mtDNA or in fixed cells labeled with an anti-TFAM antibody (Fig 6.S8B,C). Spatial analysis of pico green puncta revealed that actin inhibition promotes non-uniform distribution of mtDNA (Fig 6.4E,F).

6.2 Discussion

Here, using multiple cell types and convergent light microscopy techniques, we find that the actin cytoskeleton is a critical regulator of mitochondria distribution through mitosis. Contrary to previous investigations, we find that mitochondria in numerous cell types are highly motile throughout cell division. We determined that this motility was not dependent on microtubules, but rather on the actin cytoskeleton. Specifically, we identified a periodic, traveling wave of sub-cortical actin filaments, that propagates through metaphase mitochondria networks. This CDC42-N-WASP-Arp2/3 dependent actin “cloud” drives transient bursts of mitochondrial motility which facilitate the homogenous distribution of mitochondria throughout metaphase cells. Inhibition of the metaphase actin cloud promotes clumped and inhomogeneous mitochondria networks and prevents the spatial mixing of mtDNA nucleoids prior to division.

Previous investigations have introduced both passive and active models to explain mitochondria network inheritance. Integrating our observations with the established literature, we propose a hybrid model, which we call the “cytoskeletal handoff model”. Specifically, we propose that mitochondria fragment and release from microtubule tracks in prophase (Chung et al., 2016). From prophase through metaphase, mitochondrial motility is coordinated by the actin cytoskeleton. Specifically, a cycling wave of actin
filaments drives bursts of mitochondrial movement, ensuring that the mitochondria network is mixed and evenly distributed throughout rounded, three dimensional cells. Finally, in early anaphase, the now shuffled and homogenously distributed mitochondria re-associate with growing astral microtubules and are actively targeted to daughter cells (Kanfer, 2015).

There are several reasons why an energetically costly actin-based mixing mechanism could be advantageous. First, this mechanism of motility ensures even spacing of mitochondria and prevents organelle clumping, much like stirring a boiling pot of spaghetti prevents the noodles from sticking together. Second, actin cycling distributes mitochondria throughout the entire three-dimensional cell. As mitochondria release from microtubules before mitotic rounding is complete, actin-based motility may be required to drive the upward motility of mitochondria. As cell division is an energetically costly process, the homogenous distribution of mitochondria throughout rounded cells may be essential to locally provide ATP. Third, metaphase actin waves shuffle the spatial localization of mitochondria before division. Mitochondria in different regions of the cell are exposed to unique subcellular niches and potentially experience different levels of damage. Actin clouds shuffle the entire mitochondria network so that each daughter cell inherits a representative sample of mitochondria and not simply the 50% of organelles that happened to be on one side of the cell in interphase.

Further, this mechanism of stochastic shuffling may ensure that levels of mtDNA heteroplasmy remain constant between daughter cell lineages. In interphase cells, mitochondria are elongated and contain numerous mtDNA nucleoids. Spontaneous mtDNA mutations that occur during mtDNA replication will first expand within a single
mitochondrion, such that all the nucleoids contain mutated mtDNA. In mitosis, elongated mitochondria fragment such that each organelle has on average 1-2 nucleoids. Without an active mechanism to separate mitochondria containing mutant mtDNA there is a disproportionate chance that they co-segregate and increase levels of heteroplasmcy in the daughter cell. Thus, actin clouds ensure that mtDNA nucleoids that were spatially proximate in interphase have the potential to be randomly segregated to either daughter cell.
6.3 Figures

6.3.1 Fig. 6.1.

Fig. 6.1. Mitochondria network reorganization in m-phase. (A, top row) Maximum intensity projection of mitochondria (mito-DsRed2) in interphase (blue) and metaphase (orange) HeLa cells. (A, bottom row) Orthogonal XZ projection of HeLa mitochondria. (B) Comparison of mitochondrial orientation between interphase (left) and metaphase (right) cells. (C) Quantitation of mitochondrial orientation relative to the substratum in interphase (left), metaphase (center), and nocodazole treated interphase cells (right). (D,E) XZ projections and 3D renderings of mitochondria in interphase (D) and metaphase (E) cells. (F) Cumulative projection of mitochondria (mito-DsRed2) in nocodazole treated metaphase cell (25 µM, 90 min). Scale bars, 10 µm [A,F], 5 µm [B].
6.3.2 Fig. 6.2.

Fig. 6.2. Actin clouds cycle through metaphase mitochondria networks. (A) Lattice light-sheet rendering of mitochondria (mito-DsRed2; blue) and F-actin (EGFP-Lifeact; orange) in a metaphase HeLa cell. (B) Orthogonal views of mitochondria (mito-DsRed2; blue) and F-actin (AF488 Phalloidin; orange) in a fixed HeLa cell. Dashed line indicates location of mitochondrial actin cloud. (C) Confocal time lapse of actin (mScarlet-Lifeact) and mitochondria (MitoTracker Deep Red) in a metaphase cell. White arrow indicates the position of the actin cloud. (D) 3D time-projection of actin signal from (C). Cortical actin was cropped to visualize the subcortical actin cloud. (E) Autocorrelation of actin wave in metaphase HeLa cells. (F) Representative examples of mitochondrial actin cloud periodicity in interphase (top) and metaphase (bottom) HeLa cells. (G) Quantitation of actin cloud period (left) and velocity (right) in interphase and metaphase cells. (H,I) Kinetics of actin assembly on mitochondria in interphase (H) and metaphase (I) HeLa cells. (J) Estimated cumulative density function comparing the FWHM of (H) and (I). Error bars = 95% conf. int. (E,H,I,J). Scale bars, 10 µm [A,B,C].
Fig. 6.3. Actin clouds promote mitochondrial motility. (A-C) Percent of cells with visible actin clouds after treatment with indicated inhibitors (A,C) or siRNA (B). (D) Cumulative time projection of mitochondria signal in metaphase cells transfected with either control siRNA (top; 40nM, 48h) or Arp3 siRNA (bottom; 40nM, 48h). (E) Representative binary mask of mitochondria signal in frame 1 and in the cumulative time projection for control (top) or Arp3 (bottom) siRNA treatment. (F) Cumulative time projection of mitochondria signal in untreated cells, or cells incubated with 100nM Cytochalasin D for 1h. (G) Representative binary masks of mitochondria signal in frame 1 and in the cumulative time projection for untreated (top) or Cytochalasin D treated cells (bottom). (H) Five-minute mitochondrial displacement index in metaphase cells. (I) Mitochondria area of metaphase cell in single confocal slice. (J) Cytoplasmic area of metaphase cell in single confocal slice. (K) Mitochondrial coverage of cytoplasmic area over five minutes. (L) Histogram of peak mitochondrial velocity for actin-positive and actin-negative mitochondria in metaphase cells. Scale bars, 10 μm [D,C]. Mean ± S.D. [A-C,H-K].
Fig. 6.4. Actin cycling ensures homogenous mitochondrial distribution. (A) Focal activation of mito-GFP in LifeAct-mScarlet expressing cell metaphase cell. Kymograph of Lifeact (top; orange), mito-GFP (center; blue), and both together (bottom). (B,C) Orthogonal maximum intensity projection of mitochondria (mito-DsRed2) and actin (EGFP-LifeAct) in cells treated with DMSO (B) or wiskostatin (C). (D) 2µm thick maximum intensity projection of pico green labeled mtDNA in DMSO (top) or Cytochalasin D (bottom) treated metaphase cells. (E) Mean nearest neighbor distance (NND) between mtDNA nucleoids in cells treated with DMSO, Cytochalasin D, or Latrunculin B. (F) Index of dispersion of mtDNA nucleoids for the indicated treatments. Scale bars, 10 µm [A]. Mean ± S.D. [E,F].
Fig. 6.S1. Mitochondrial orientation shifts in mitosis. (A) 3D projection of LifeAct-EGFP expressing HeLa cell undergoing mitotic rounding. Over 27 minutes, the cross-sectional area of the cell decreases (top row) while the cell increases in height by a factor of 3 (bottom row). Dashed line indicates cell boundary. White arrows indicate apical surface of the cell. (B) XZ projection of mitochondria in a metaphase HeLa cell with enlarged inset (yellow box). (C-F) Orthogonal projections of mito-paGFP in interphase cells (C,D) and metaphase cells (E,F). (G) Orthogonal projections of mitochondria (mito-DsRed2) in A549 cells. Scale, 10 µm [A,B,E-G]; 5 µm [C,D].
Fig. 6.S2. Mitochondrial motility in metaphase. (A) Maximum intensity projections of mitochondria movement over five minutes in HaCaT, HEK293T, and NHEK cells. Projections are pseudocolored based on time. Dashed lines indicate the perimeter of metaphase cells. White arrows indicate highly dynamic regions of the mitochondrial network. (D) Cumulative time projections indicating metaphase mitochondria motility in the indicated cell lines. (E,F) isIM images of mitochondria (HSP60) and microtubules (alpha tubulin) in interphase (E) and metaphase (F) HeLa cells. Scale, 10 μm [A-D, E top row, F, top row], 2.5 μm [E bottom row, F bottom row].
Fig. 6.3.7 Fig. 6.S3.

Actin assembles on mitochondria in mitosis. (A) iSIM image of mitochondria (mito-DsRed2) and F-actin (LifeAct-EGFP) in a live, metaphase HeLa cell. (B) Spinning disk image of mitochondria (Tom20, blue) and F-actin (Phalloidin, orange) in a fixed, metaphase HeLa cell. Merged image shows spindle (alpha-tubulin, cyan) and aligned chromosomes (Hoechst, blue). Yellow arrows indicate actin positive mitochondria. (C) Timelapse of Lifeact intensity in a metaphase HeLa cell over 12.5 minutes. White arrows indicate position of the actin cloud. Actin intensity within dashed box is shown below. (D) Angular velocity of actin clouds in metaphase HeLa cells. (E) Directionality of actin clouds in metaphase HeLa cell. Bidirectional indicates cells with two actin clouds moving in opposite directions. (F) Percentage of cells with visible actin clouds in each stage of mitosis. (G) Spinning disk image of F-Actin (phalloidin, orange) and mitochondria (Tom20, blue) in a fixed, telophase HeLa cell. Microtubules (alpha tubulin, cyan) and DNA (Hoechst, blue) are shown for reference. Scale, 10 µm [A top, B top, C top, G top], 5 µm [G bottom], 2.5 µm [B bottom].
Fig. 6.S4. Interphase actin assembly on mitochondria. (A, left) Maximum intensity projection of actin (LifeAct-EGFP, green) and mitochondria (mito-DsRed2, magenta) in a live, interphase HeLa cell. (A, center) Inset showing actin positive mitochondria. (A, right) Orthogonal projections of actin and mitochondria. (B) Airy-scan image of actin (LifeAct-EGFP, green) and mitochondria (mito-DsRed2, magenta) in a live, interphase HeLa cell. Insets indicate actin positive mitochondria (top) and actin negative mitochondria (bottom). (C) Autocorrelation of interphase actin wave. (D) Representative trace of actin intensity on mitochondria over time. (E) Directional tracking of interphase actin cloud over time. Traces that trend to the left indicate a counterclockwise cycling bias, while traces to the right indicate a clockwise cycling bias. (F) Quantitation of actin cloud speed in cells plated on crossbow, disk, or H shaped micropatterns. (G) Representative image of HeLa cell plated on crossbow pattern. (H) Representative trace of actin intensity over time in HeLa cell plated on crossbow micropattern.
Fig. 6. S5. Filamin A and Alpha-Actinin associate with mitochondrial actin clouds. (A) Cartoon model depicting the mechanism of actin cloud assembly. (B) Spinning disk confocal images of mitochondria (mito-DsRed2, blue), F-actin (LifeAct-EGFP, magenta), and Filamin A (Emerald-Filamin A, green). (C) F-actin (Phalloidin, magenta), and Filamin A (anti-Filamin A, green) in a fixed, early anaphase HeLa cell. Blue arrows indicate colocalization of actin and Filamin A. (D) Spinning disk image of mitochondria (mito-DsRed2, blue), F-actin (LifeAct-EGFP, magenta), and Filamin A (Emerald-FilaminA, green), in a live, metaphase HeLa cell. (E) Line scan indicates colocalization of LifeAct and Filamin A around mitochondria. (F) Colocalization of Lifeact (magenta) and Alpha-actinin (mNeongreen-AlphaActinin, green) on mitochondria (mito-DsRed2, blue). (G) Colocalization of actin cloud (EGFP-LifeAct, magenta) and cortactin (Cortactin-EGFP, green).
6.3.10 Fig. 6.S6.

Fig 6.S6. Actin waves promote mitochondrial motility. (A, left) Spinning disk image of F-actin (LifeAct-mScarlet, green) and mitochondria (MitoTracker Deep Red, magenta), in a metaphase HeLa cell. Yellow arrow indicates line scan used for kymograph. (A, right) Kymograph of actin (top) and mitochondria (bottom). Blue arrows indicate sites where actin waves displace mitochondria. (B) Cumulative time projections of mitochondria (mito-DsRed2, orange) movements over 5 minutes in cells treated with Latrunculin B (top, 10µM, 1h) or C3 Transferase (bottom, 3µg/ml, 2h). (C) Time-lapse of mitochondria (MitoTracker Deep Red, orange) and ER (GFP-Sec61B, gray) in a metaphase HeLa cell. The motile, actin-positive mitochondrion is pseudocolored yellow for visualization purposes. Dashed yellow line indicates position of the motile mitochondrion relative to the ER tubules. (D) Spinning disk confocal image of mitochondria (MitoTracker Deep Red, orange) and ER (EGFP-Sec61B, cyan) in HeLa cell treated with Nocodazole (25 µM, 1h). Orthogonal show mitochondria association with ER it the XZ plane.
Fig. 6.7. Actin clouds promote mitochondria movement in the absence of microtubules. (A) Cumulative time projection of actin positive mitochondria (top) and actin negative mitochondria (bottom) in an interphase HeLa cell treated with nocodazole (25 µM, 1h). (B) Quantitation of peak mitochondrial velocity for actin-positive and actin-negative mitochondria in the indicated conditions.
**Fig. 6.S8.** Metaphase actin clouds ensure homogenous distribution of mitochondria. (A) Orthogonal projections of actin (LifeAct-EGFP) and mitochondria (mito-DsRed2) in cells treated with the Rho GTPase inhibitor C3 Transferase (3 µg/ml, 2h). (B) Spinning disk confocal image of F-actin (Phalloidin, orange) and mtDNA (TFAM, blue) after 1h incubation with DMSO or Cytochalasin D (100nM). (C) Distribution of mtDNA nucleoids as visualized in live cells with SNAP TFAM (left) or fixed cells with anti-TFAM antibody (right). (D, model; left) In normal cells, metaphase actin clouds (green arrow) distribute focally damaged mitochondria (orange) throughout the entire mitochondria network. Consequently damaged mitochondria are equally inherited between daughter cells. (D, model; right) Upon inhibition of actin cycling, focally damaged mitochondria are not mixed throughout the network. Thus, these damaged organelles are inherited entirely by one daughter cell.
CHAPTER 7: DISCUSSION

The three projects described in this thesis address distinct aspects of mitochondria network homeostasis.

7.1 Mitophagy

Since the discovery of PINK1/Parkin mitophagy in 2008 (Narendra, 2008), dozens of research groups have worked to piece together the molecular details of this complex pathway. The work that I presented in Chapter 3 uses multi-color live imaging to establish the kinetics of mitophagy and contributes detailed information about the regulation of autophagy receptors by the serine threonine/kinase TBK1. One major strength of the investigation was our discovery that a subset of ALS-associated mutations in OPTN and TBK1 delay the autophagic engulfment of acutely damaged mitochondria. Based on this finding, we reasoned that inefficient recognition of damaged mitochondria could potentially play a role in ALS pathogenesis.

However, the impact of our investigation was limited by two main factors: (1) the experimental model system and (2) the damage paradigm we used to trigger mitophagy.

All of the experiments performed in Chapter 3 were carried out in HeLa cells, a human cervical adenocarcinoma line that has been continuously cultivated since 1951. There are a number of excellent reasons to use HeLa cells (outlined later in this section), but it is possible – maybe even probable – that mechanisms of PINK1/Parkin mitophagy are not equivalent between HeLa cells and neurons. Additionally, we used highly artificial mechanisms to trigger mitochondrial damage (see chapter 2). Though we were careful to
use at least three distinct mitochondria damage paradigms, the fact remains that neither chemical depolarization nor intense laser irradiation represent physiologically relevant mitochondrial stressors. Whether cellular responses to these types of acute stressors are equivalent to those used in response to pathophysiological stressors observed in disease states is not clear. Many of the major discoveries in the PINK1/Parkin field have been made using these models (Narendra, 2008; Lazarou, 2015). However, now that the PINK1/Parkin pathway is better understood, a major goal of the field should be ascertaining whether this form of mitophagy is relevant \textit{in vivo}, and not simply a characteristic of cancer cells or transformed cell lines.

7.1.1 Mitophagy \textit{in vivo}

At least five genes involved in the PINK1/Parkin pathway have been linked to neurodegeneration in humans, suggesting that this type of organelle turnover is critically important to human health. However, the necessity of PINK1/Parkin mitophagy in living organisms is controversial.

Multiple groups have generated mouse lines that can be used to measure mitophagy \textit{in vivo} (Sun et al., 2016; McWilliams et al., 2016). The Mito-QC mouse expresses a transgene encoding an mCherry-EGFP tandem tag fused to a mitochondrial outer membrane targeting sequence (McWilliams et al., 2016). Under normal circumstances, mitochondria will be visualized by both red and green markers, however, when mitochondria are targeted to acidic compartment such as a lysosome, the EGFP fluorescence is quenched and the mitochondria will only be labeled by a red marker. The similar mt-Keima mouse expresses a mitochondria-targeted Keima protein, which
fluoresces green at neutral pH, but shifts to red once acidified in lysosomes (Sun et al., 2016). Both Mito-QC and mt-Keima mice can be used to estimate basal rates of basal mitophagy in different tissues.

In a follow up to their original paper, McWilliams and colleagues (2018) crossed their Mito-QC reporter line with PINK1 knockout mice. Strikingly, the investigators determined that elimination of Pink1 had no effect on basal levels of mitophagy. Mito-QC and mt-Keima Drosophila lines similarly showed that loss of either Pink1 or parkin had little to no effect on basal rates of mitophagy (Lee et al., 2018).

Even if PINK1 and Parkin are dispensable for basal rates of mitophagy, this does not necessarily indicate that PINK1/Parkin-dependent mitophagy does not occur in vivo. A simple explanation may be that this form of selective mitophagy represents a “backup”, fail-safe type of mitochondrial quality control that is only activated upon extreme stress or once other QC mechanisms become overtaxed. Consistent with this hypothesis PINK1 and Parkin knockout mice fail to develop Parkinson’s disease symptoms over their ~2 year lifespan (Goldberg et al., 2003; Zhou et al., 2007; Kitada et al., 2007; Kitada et al., 2009). However, when Parkin knockout mice are crossed with “mutator mice,” a mouse line that accumulates mtDNA mutations at an accelerated rate, the progeny display robust mitochondrial dysfunction and degeneration of dopaminergic neurons (Pickrell et al., 2015). Indeed, the idea that PINK1/Parkin mitophagy is only activated in response to extensive mitochondrial damage may explain why individuals with mutations in these genes do not develop Parkinson’s disease until midlife, at which point their mitochondria have been exposed to decades of environmental stressors.
PINK1 and Parkin are ubiquitously expressed, but loss of function mutations in these genes result in selective neurodegeneration. It is not entirely clear why these mutations do not also result in degeneration of other metabolically active tissues, such as heart or liver. One possibility is that neurons possess a number of unique characteristics that enhance their sensitivity to mitochondrial damage: (1) neurons rely almost exclusively on mitochondrial metabolism for their energy production (Gusdon & Chu, 2011; Federico et al., 2012). In contrast to proliferative cell lines that can survive on glycolysis alone (Gogvadaze et al., 2010; Bolanos et al., 2010), neurons require a highly efficient mitochondrial network to carry out their core homeostatic and electrophysiological functions. Thus, neurons might possess regulatory mechanisms to downregulate mitophagy in order to maintain sufficient mitochondrial mass to survive. (2) Neurons are highly polarized cells that can stretch over several meters in length in some species. This extreme size allows cells to eclectically transmit information over long distances, but also necessitates a vast and sprawling mitochondrial network (Schwarz, 2013). Whether mitochondria occupying distal axonal regions are subject to the same quality control mechanisms as those near the cell body remains controversial (Ashrafi et al., 2014; Cai et al., 2011; Sheng et al., 2012; Lin et al., 2017). (3) Neurons are most-mitotic and must maintain healthy mitochondria for an entire lifespan. Consequently, mature neurons cannot eliminate their damaged mitochondria through asymmetrical partitioning during cell division, as has been proposed for proliferative cells.
7.2 Mitochondrial actin clouds

My project examining mitochondrial autophagy (Chapter 3) led to a collaboration with a senior graduate student, Yvette Wong, who was interested in understanding the role of actin assembly in autophagosome formation. However, the focus of our collaboration quickly shifted when we observed a wholly unexpected and previously uncharacterized phenomenon (Chapter 5). Using live-cell imaging we identified a cloud of actin filaments propagating through interphase mitochondria networks at a rate of ~5µm/min. This actin cloud associates with 20% of mitochondria at a given time, where it transiently promotes mitochondrial fission. After 3-5 minutes, the cloud disassembles, mitochondria fuse back together, and the cloud reassembles on a neighboring region of the mitochondrial network, where the process repeats. We hypothesized that cycling actin clouds function as constitutive regulators of mitochondrial length and prevent mitochondrial hyperfusion which is linked to cell senescence.

This work identified and characterized a novel mechanism by which mitochondrial networks maintain their morphology. Further, it challenged the accepted notion that mitochondrial network morphology is regulated by a dynamic equilibrium between fission and fusion at the level of the individual organelle, and instead suggested that rapid, actin-dependent regional remodeling controls mitochondrial network architecture.

Below, I outline several techniques and best practices for imaging mitochondrial actin assembly. Using these techniques, I have visualized actin clouds in HeLa, Cos7, HEK293T, HaCaT, A549, MCF-7, and IPS cells.
7.2.1 Choosing appropriate fluorescent probes

I have successfully visualized actin clouds using fluorescent fusions of LifeAct, F-Tractin, and the calponin homology domain of Utrophin (Utr-CH). I have also found that it is possible to track actin clouds with fluorescent actin (b-actin-GFP) or the far-red dye SiR-Actin, but in both cases the signal-to-noise ratio of actin clouds is quite low. In HeLa cells, it appears that Utr-CH (mScarlet-I-UtrCH; addgene 98823) best approximates phalloidin staining of F-actin but is also the most toxic to cells. In contrast, LifeAct appears to be largely non-toxic, and I have successfully visualized actin cycling with numerous LifeAct fusions, including LifeAct-TagBFP2, LifeAct-EGFP, LifeAct-mNeonGreen, LifeAct-mRuby, LifeAct-mScarlet, LifeAct-TagRFP-T, LifeAct-HaloTag, and LifeAct-miRFP703.

7.2.2 Use of stable cells

Transient transfection, whether by chemical, electrical, or mechanical means, is a relatively imprecise procedure that can result in heterogeneous expression patterns across cell populations. Extreme overexpression of LifeAct alters actin dynamics (Courtemanche, 2016) while high levels of mitochondria matrix marker have been linked to mitochondrial fragmentation (Mitra, 2010). Thus, generation of stable cell lines is the best option for long term visualization of mitochondrial actin clouds. Lentiviral transduction coupled with fluorescence activated cell sorting is a simple procedure to engineer monoclonal HeLa lines expressing actin and mitochondrial markers. Once a monoclonal line is generated, quality control steps can be carried out to ensure the cells are dividing and growing normally, and there are little or no overexpression artifacts.
7.2.3 *Appropriate microscopy techniques*

Mitochondrial actin clouds are most easily visualized by confocal microscopy. In HeLa cells, actin clouds are typically clearest between 2-8 µm from the basal surface of the cell. Without the use of a confocal pinhole to eliminate out of focus light, the signal from dense cortical actin structures makes it extremely challenging to resolve actin clouds. Even within single optical sections, actin clouds are often difficult to distinguish from brighter actin structures, such as stress fibers or lamellipodia. Due to the speed of actin cycling, spinning-disk confocal microscopy is an ideal imaging modality, though I have successfully imaged actin cycling using fast superresolution techniques, such as Zeiss AiryScan and VisiTech iSIM.

7.3 *Studying mitochondria dynamics in cell lines*

7.3.1 *The case for cell lines*

Immortalized mammalian cells have served as a work horse for organelle biology studies. These models have a number of ideal characteristics that make them suitable for questions of mitochondrial structure. (1) These cells are inexpensive, easy to culture, and typically have large dynamic mitochondrial networks that can be easily labeled by either dyes (MitoTracker, TMRE, Jc-1, etc.) or genetic approaches (transfection of fluorescent mitochondrial marker). (2) Between 3µm and 10µm in height, immortalized mammalian cells are optically clear and can be easily mounted and imaged on a standard widefield microscope. (3) Generation of stable cell lines is a simple and reproducible procedure. (4) Immortalized cells are ideal for high content imaging screens of small molecule inhibitors, RNAi, or CRISPR. (5) There is already a large established literature on mitochondrial dynamics in cell culture. Indeed, visualization of mitochondria in mammalian
cell culture stretches back over a hundred years to the pioneering work of Margaret and Warren Lewis (Lewis and Lewis, 1915).

7.3.2 The case against cell lines

The use of mammalian cell culture makes practical sense: as described above, mammalian cell lines are widely used, easy to culture, genetic tractable, and optically accessible. However, immortalized cell lines have several undesirable characteristics with respect to understanding physiologically relevant mitochondrial biology. (1) Cancer cell lines carry out aerobic glycolysis (Warburg metabolism) and show reduced reliance on their mitochondria for energy generation. Indeed, HeLa cells can survive for prolonged periods of time with mitochondria containing no mtDNA (and even without mitochondria whatsoever). (2) Many of the cell lines commonly used for mitochondrial studies, such as U2OS and HeLa, are hyperploid and have expression profiles that often differ significantly from real tissues in the human body. Complicating matters further, cell lines in two neighboring labs may have very different expression profiles due to genetic drift or differences in culture conditions (Indeed, we found that the HeLa cells used for the experiments in Chapter 3 had different expression levels of OPTN as compared to HeLa cells obtained from the Youle lab, potentially complicating interpretation of mitophagy results). (3) Cultured cells are often cross-contaminated by other cell lines or infected with mycoplasma, both of which can confound cell biological investigations and lead to irreproducible results. (4) The two-dimensional glass environment of a cell culture dish fails to model the mechanical environment that a given cell would experience in vivo. As the cellular environment is an important determinant of the cytoskeleton and thus the distribution and morphology of mitochondria, this represents a major shortcoming of 2D cell culture. This caveat applies not only to immortalized cancer cells but also to primary
cells such as rodent cortical or hippocampal neurons, which have been widely used to study mitochondrial dynamics and turnover. (5) Small changes in cell confluence, passage number, media composition or pH, and media temperature can rapidly affect mitochondrial dynamics and morphology.

7.3.3 Future steps

Many of the rationales for using immortalized tissue culture cells over more physiologically relevant systems such as induced pluripotent stem cells (iPSCs), primary cells, organoids, or model organisms, are disappearing with the advent of new technologies. CRISPR/Cas9 has simplified the process of generating tissue specific/inducible knockout model organisms (of note CRISPR has not been shown to efficiently edit mtDNA). Further, new selective plane illumination microscopy (SPIM) techniques are allowing for rapid, high resolution imaging of 3D environments such as organoids, Drosophila brains, or c. elegans embryos. Moving forward, I believe that it is critically important to investigate mitochondrial actin cycling in a more physiological context. Specifically, two-photon microscopy of transgenic Lifeact-GFP and mito-DsRed2 expressing mice would shed light on whether cycling actin clouds are an in vitro artifact or a bona fide in vivo mechanism of mitochondria network maintenance.

7.4 Emerging areas of mitochondrial research

7.4.1 Mitochondria-Organelle contacts

One of the major emerging topics in mitochondrial biology is the functional interplay between mitochondria and other membranous organelles. To date, mitochondria have been shown to associate with plasma membrane, lysosomes, lipid droplets, Golgi
apparatus, vacuoles, peroxisomes, and, most notably, the endoplasmic reticulum (Murley & Nunnari, 2016). These contact sites are often transient but play important physiological functions. Mitochondria-ER contact sites, or mitochondria associated membranes (MAMs), drive lipid and ion transfer (Rizzuto et al., 2009), signaling (Ishikawa & Barber, 2008), and have been shown to mark prospective sites of mitochondrial fission (Friedman et al., 2011).

Because mitochondria-organelle contacts can be extremely transient, effectively imaging and quantifying these associations has been challenging. Work from the Lippincott-Schwartz lab paired multispectral imaging with lattice light sheet microscopy to volumetrically image six organelles in live cells and track their associations (Valm et al., 2017). This method offers a more complete picture of mitochondrial/endomembrane contacts but generates enormous data sets that may be difficult to interpret. Further, it relies on the use of dyes or the overexpression of several organelle markers, which can potentially affect organelle homeostasis. The development of split FPs or FRET sensors offers an alternative mechanism to study inter-organelle contacts (Kakimoto et al., 2018). Finally, electron microscopy offers nanometer-scale insight into the organelle landscape, but the technique can only be used on fix tissues and is difficult to multiplex with light microscopy.

7.4.2 A mitochondrial atlas

A second major direction of recent mitochondrial research has been an attempt to map the mitochondrial proteome. Despite over 100 years of mitochondrial research, the full complement of mitochondrially localized proteins is still not fully known. New techniques for enzyme mediated proximity labeling have generated comprehensive lists of putative
mitochondrial proteins, often identifying surprising hits never before linked to mitochondria. These techniques, which include BioID (Roux et al., 2012) and APEX (Martell et al., 2012) work by targeting an engineered enzyme to a specific region of the mitochondrion, where, upon addition of certain substrates, biotinylate and label all nearby proteins. Biotinylated proteins can then be identified visually, by western blotting, or by mass spec analysis. BioID and APEX have been used to identify candidate proteins in mitochondrial matrix (Rhee et al., 2013), intermembrane space (Hung et al., 2014), inner and outer membranes (Lee et al., 2016), as well as mtDNA (Liyanage et al., 2017; Han et al., 2017);

Upon identifying the complete mitochondrial proteome, an important next step will be to spatially map the localization of each protein within its compartment. However, conventional light microscopy techniques cannot resolve mitochondrial proteins that are within ~250 nm. Consequently, superresolution light microscopy techniques, including SIM, STED, PALM/STORM, and expansion microscopy can be used to precisely localize mitochondrial proteins. These superresolution techniques can then be correlated with electron microscopy images which give nanometer details of mitochondria membrane ultrastructure.
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