Regulation of Mitochondrial Distribution and Inheritance During Cell Division

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Regulation of Mitochondrial Distribution and Inheritance during Cell Division

A dissertation presented

by

Jarom Y Chung

to

The Division of Medical Sciences

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Regulation of Mitochondrial Distribution and Inheritance during Cell Division

Abstract

Mitochondria are crucial to the cell and perform numerous functions including generating cellular ATP, buffering calcium, and creating macromolecules. Mitochondria contain their own DNA and as such, cannot be synthesized de novo. Additionally, both nuclear-encoded and mitochondrial-encoded proteins work in concert in order for mitochondria to function. During cell division, the cell goes through dramatic morphological changes to ensure the appropriate amount of DNA is inherited into daughter cells. Given the importance of mitochondria, our study sought to understand the underlying mechanisms that ensure proper mitochondrial inheritance.

Organelles are inherited by two mechanisms: active and passive. Through our studies we found that mitochondria go through phases of both active and passive regulation. Initially, mitochondria undergo a release from microtubules, actin, and the endoplasmic reticulum, which allows mitochondria to passively float throughout the cytoplasm. As the cell enters cytokinesis, an active phase occurs that can compensate for some, but not all, asymmetry prior to cytokinesis. The detachment from microtubules, actin, and ER is regulated during cell division, and we discovered the mechanism behind mitochondrial release from microtubules. Dynein and kinesin motors move mitochondria along microtubules and also attach them to the cytoskeleton. During mitosis, motors shed from the mitochondrial surface, thus releasing mitochondria from microtubules. CDK1 releases dynein through phosphorylation, and Aurora A kinase releases kinesin. When exogenously expressed motors are recruited to mitochondria, it results in
asymmetric inheritance of mitochondria, a delay in mitotic progression, and cytokinesis failure. Since mitochondria are initially positioned passively, we were able to manipulate mitochondrial positioning before the onset of mitosis, which persisted into the duration of mitosis.

Overall, our study elucidates the mechanism behind mitochondrial inheritance. Undoubtedly, the mechanisms are important for normal inheritance into daughter cells. It remains an open question of whether or not directed inheritance can take place through these same mechanisms.
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Dedication

To Megan, Jinora, and all my future little ones: life is an exciting series of experiments. My PhD taught me that experiments are hard, they will fail, they won’t always give you the results you want, and they require work to figure out the perfect conditions, but sometimes they work the way you want and you learn something interesting and beautiful. You have to keep trying and keep believing that you’ll figure out the answer some day.
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Chapter 1: Introduction
Organelle Inheritance during Mitosis

During the course of a cell’s life, it proceeds through distinct phases of growth and synthesis in preparation for mitosis when the cell divides into two daughter cells. Broadly speaking, a cell’s life can be broken down into two main phases: interphase and mitosis. Much of the study of mitosis has focused on segregation of chromosomes into daughter cells. However, DNA is but one of the many elements the cell needs to function. In particular, organelles are required for a fully functioning cell. To that end, the question arises of how the cell accurately and appropriately ensures “proper inheritance” of important cellular materials into daughter cells.

Figure 1.1

Active Segregation

Passive Segregation

Cell Growth Duplication

Cell Division

Figure 1.1 Diagram of how organelles are inherited during mitosis.
Organelle inheritance can be classified into two categories: active and passive (Figure 1.1). Active inheritance is an ordered segregation strategy that requires the organelle of interest to be tethered and/or transported purposefully and deliberately. It leaves no room for error due to random chance; rather, it ensures precision during segregation. Active inheritance is best exemplified by chromosome inheritance (McIntosh and Koonce, 1989). Each chromosome aligns along the metaphase plate and attach to kinetochore microtubules, which emanate from spindle poles. Once each chromosome is aligned and attached, the cell passes through the spindle assembly checkpoint, and the cell separates the daughter chromatids into what will be the two daughter cells. The process of inheriting the appropriate amount of DNA is actively regulated by microtubule attachment as well as various checkpoints to ensure the fidelity of the process. Other known actively inherited organelles are the centrosomes, which make up the two spindle poles and are segregated precisely so that each daughter cell inherits exactly one copy.

Passive inheritance is a stochastic form of segregation and assumes the organelles are not attached to any tether that would ensure precise segregation. Typically, organelles that appear in large numbers are thought to go through passive inheritance. With a large number of particles, there is a higher probability of accurate inheritance (Birky, 1983). Likewise, a large quantity would create even and random distribution throughout the cytoplasm. Passive inheritance also suggests that an equal volume of the cell’s cytoplasm is inherited into each of the daughter cells (Bergeland et al., 2001). Mitochondria, chloroplasts, endoplasmic reticulum (ER), Golgi, vesicles, lysosomes, and peroxisomes are thought to be inherited passively given that they fulfill the previous criteria. Large organelles like Golgi, ER, and mitochondria go through massive remodeling to decrease their size and increase their number during cell division; whereas, small organelles like endosomes, lysosomes, and peroxisomes already exist in high number and are
primed for cell division (Cerveny et al., 2007; Dunster et al., 2002; Jesch et al., 2001; McCullough and Lucocq, 2005; Rabouille and Jokitalo, 2003; Rabouille and Kondylis, 2007; Shima et al., 1998; Sutterlin and Colanzi, 2010).

Passive and active inheritance are not mutually exclusive as it was once thought to be. Although most organelles detach from microtubules and are actively excluded from the mitotic spindle, some colocalize with cytoskeletal elements (Cascone et al., 2008; Dunster et al., 2002). For instance, peroxisomes, ER, and Golgi apparatus have characteristics of both active and passive inheritance. The ER releases from microtubules through STIM1 phosphorylation which disrupts binding to EB1, a known microtubule-associated protein. Although the majority of ER floats passively throughout the cytoplasm, subdomains interact with the cortical actin shell and the microtubule-organizing center (Smyth et al., 2012). Similarly, peroxisomes and the Golgi apparatus interact with the microtubule-organizing center but also are spread throughout the cytoplasm (Jesch et al., 2001; Kredel et al., 2009; Sutterlin and Colanzi, 2010). On the other hand, mitochondrial distribution and mode of inheritance during mitosis remain a mystery. Does mitochondrial inheritance occur through passive or active means? If mitochondria are inherited passively, how do they detach from their normal tethers?

**Mitochondrial fission and fusion dynamics**

The importance of mitochondria has long been known primarily with regards to providing energy via oxidative phosphorylation, buffering calcium to maintain cellular homeostasis, synthesizing macromolecules and lipids, and producing reactive oxygen species. Given their importance, there are numerous mitochondria within the cell that are spread throughout the cytoplasm.
Mitochondria function through a combination of nuclear-encoded and mitochondrial-encoded proteins. Mitochondria contain their own DNA (mtDNA, nucleoids), which replicates by mitochondrial specific machinery. Likewise the unique inner and outer membrane structure is regulated in order to maintain membrane potential for oxidative phosphorylation. These distinct subdomains—the inner and outer membranes—are maintained as mitochondria divide.

Mitochondria divide and fuse during the cell cycle. During growth phases (G1/G2), the mitochondria are a mixture of short, round mitochondria as well longer, tubular mitochondria that are often interconnected. During the DNA synthesis phase (S), the mitochondrial network becomes fused (Mitra et al., 2009). During cell division, the mitochondrial network goes through extensive fission to increase its numbers. Mitochondrial fission depends heavily on the dynamin-related GTPase, DRP1, which is recruited to the mitochondrial surface by a mitochondrial receptor MFF (Liu and Chan, 2015). To provide the constriction forces, actin polymerization is required between the ER and mitochondrial fission sites through the actin-regulating proteins INF2 and myosin IIA/B (Ji et al., 2015; Korobova et al., 2014; Korobova et al., 2013). During mitosis, DRP1’s activity increases through a coordinated effort of mitotic proteins. The mitotic kinase Aurora A phosphorylates the small Ras-like GTPase RALA and localizes it to the mitochondrial surface. RALA’s cofactor RALBP1 is recruited along with cyclin B-CDK1, bringing this important kinase to the mitochondrial surface. Last, CDK1 phosphorylates DRP1 and increase its activity (Taguchi et al., 2007). Through this regulation, the overall number of individual mitochondria increases, thus creating an increased probability of equal inheritance through passive means.

In addition to up-regulating the fission apparatus, the cell also down-regulates mitochondrial fusion dynamics. During interphase, mitofusin 1 (MFN1) homodimerizes with
adjacent mitochondria to fuse the two outer membranes together. During mitosis, MARCH5, an E3-ubiquitin ligase that is localized to the outer membrane of the mitochondria, ubiquinates MFN1, and targets it for degradation (Park and Cho, 2012). Although the exact mechanism is not known, MFN1 can interact with the cyclin B-CDK1 complex during the G2 and likewise during mitosis.

**Mitochondrial transport using microtubule highways**

Given their importance to the cell, mitochondrial distribution is actively regulated. Transport and mitochondrial dynamics are closely linked during the cell’s life. Mitochondrial transport was first studied using polarized cells like mitotic budding yeast and neurons. *S. cerevisiae* have polarized actin microfilaments during cell division, and neuronal axons create long, unidirectional microtubule tracks along the length of the axon.

Identification of the motor adaptor complex pioneered the field of axonal transport. In a screen designed to identify proteins important for *Drosophila* photoreceptor function, a certain mutant was identified (Glater et al., 2006). This mutant had nonfunctional photoreceptors but was morphologically intact. Upon analysis, mitochondria were clumped at the cell body, leaving the axon devoid of mitochondria. This irregular mitochondrial distribution was identified as the source of photoreceptor dysfunction. The responsible protein was later identified as a mitochondrial motor adaptor protein and was named Milton, in honor of the blind poet John Milton. An analogous screen identified the atypical Rho-GTPase Miro as another member of the motor adaptor complex. Null Miro flies also had a similar phenotype as the Milton mutants (Guo et al., 2005). Miro resides on the outer mitochondrial membrane and binds Milton, which recruits kinesin and dynein motors (Figure 1.2). Mitochondria move towards the plus ends of
microtubules via conventional kinesin-1 (KHC/KIF5A,B,C) and towards the microtubule-organizing center via dynein and dynactin (Glater et al., 2006; van Spronsen et al., 2013).

Mitochondrial motility and attachment to microtubules are primarily regulated through the motor adaptor complex. To date, mitochondrial health, calcium concentration, glucose level, and hypoxia have all been implicated in modulating mitochondrial transport (Li et al., 2009; Pekkurnaz et al., 2014; Wang and Schwarz, 2009; Wang et al., 2011). It is unclear whether the motor adaptor complex has a role in mitochondrial segregation and inheritance (Mishra and Chan, 2014).

**Figure 1.2**

![Schematic of the motor adaptor complex.](image)

*Figure 1.2* Schematic of the motor adaptor complex.
Mitochondrial inheritance in non-mammalian cells

In *S. cerevisiae*, mechanisms behind mitochondrial inheritance have been well studied compared to that of metazoans. In *S. cerevisiae*, a growing daughter cell forms as a bud off of the mother cell. Once the daughter cell reaches the appropriate size, mitochondria and other organelles move along actin cables toward the daughter via MYO2, a gene that encodes a class V myosin (Itoh et al., 2002). Machinery behind this process was first identified through a screen for cells lacking mitochondria in the daughter bud. Named the *mdm* mutants (mutants for mitochondrial distribution and morphology), these temperature-sensitive mutants ranged from defects in mitochondrial transport machinery, cytoskeleton, and proteins linking mitochondria to the ER (Fortsch et al., 2011; Higuchi-Sanabria et al., 2014; Lackner et al., 2013; Lai et al., 2002; Lewandowska et al., 2013; Westermann, 2014).

Mitochondrial segregation in *S. cerevisiae* is also dependent on mitochondrial health. Damaged mitochondria accumulate in the mother cell, and fewer mitochondria are transported into the daughter bud (Lai et al., 2002). Retrograde actin cable flow creates a treadmill effect that inhibits transport of unhealthy mitochondria into the daughter bud (Higuchi et al., 2013). Mitochondrial inheritance in *S. cerevisiae* demonstrates active inheritance. Without the proper transport machinery, the daughter cell would not inherit mitochondria. Interestingly, mitochondria from *S. cerevisiae* move along actin cables via myosin motors; whereas, other eukaryotes move throughout the cell via kinesin/dynein motors along microtubules.

In *S. pombe*, mitochondria segregation appears to be an active process as well. Mitochondria are tethered to the plus-ends of microtubules via PEG1b and MMB1p (Chiron et al., 2008; Fu et al., 2011). These proteins allow mitochondria to be distributed near the spindle poles during mitosis when the spindle elongates. Tethering to microtubules is independent of
kinesin-based motors, which is different from the kinesin and dynein dependence found in mammalian cells. In mutants lacking the microtubule-associated protein MMB1p, expression of chimeric proteins that artificially link mitochondria to kinesin or actin was unable to rescue distribution defects. The microtubule attachment is an active process that ensures even mitochondrial distribution and inheritance in *S. pombe* (Yaffe et al., 2003). Furthermore, proper distribution of mitochondria was also accompanied by semi-regular arrangement of nucleoids within those mitochondria, ensuring inheritance of mtDNA (Jajoo et al., 2016).

Similar to *S. pombe*, mitochondria in *C. merolae* have direct connections with the mitotic spindle. This physical connection was found to be insignificant for division of mitochondria but important for mitochondrial morphology changes and inheritance during cell division (Nishida et al., 2005). Last, in *Drosophila melanogaster*, it was found that mitochondria colocalize with KLP67A. This motor protein moves along microtubules toward the plus ends and colocalizes with the mitotic spindle, especially at the astral microtubules (Pereira et al., 1997). KLP67A attachment is thought to properly distribute mitochondria during cell division.

In non-mammalian cell types, mitochondria appeared to be inherited actively by tethering to microtubules filaments or being transported by actin cables. Whether or not mitochondrial inheritance in mammalian cells is an active process is still unclear (Mishra and Chan, 2014). If it is an active process, does the process involve the normal machinery that allows for mitochondria to move and distribute throughout the cell?

**Mitochondrial inheritance in mammalian cells**

In the last five years, there has been a growing interest in understanding mitochondrial inheritance particularly during cytokinesis. These reports paint a picture that inheritance relies on
attachment to cytoskeletal elements, namely actin and microtubules, but various studies have reported conflicting results. Mitochondria do not to colocalize with intermediate filaments or microtubules, but they may associate with actin filaments, although this is highly debated within the field (Lawrence and Mandato, 2013a; Lawrence et al., 2016; Lawrence and Mandato, 2013b; Lee et al., 2007; Li et al., 2015; Rohn et al., 2014). It was seen that dynein detaches from mitochondria, but it remains unclear whether or not dissociation is sufficient for microtubule release (Lee et al., 2007).

Through live cell imaging, it was seen that mitochondria move away from spindle poles during anaphase and instead move towards the cytokinetic furrow (Lawrence and Mandato, 2013b). This process may be due to a coordinate motion between microtubule and actin attachment. During one study, neither treatment of Latrunculin A, an actin depolymerizing agent, nor Jasplakinolide, an actin stabilizing agent, were able to prevent mitochondrial movement towards the midline. However, when microtubules were depolymerized with nocodazole, mitochondria were unable to move into the cleavage furrow, suggesting that the movement is through microtubules and not through actin.

CENP-F protein recruits to mitochondria in a cell cycle dependent fashion. CENP-F is a 367 kDa coiled-coil protein whose levels increase as the cell proceeds into mitosis. It has several functions, but importantly, it interacts with dynein and microtubules. CENP-F binds the mitochondrial adaptor Miro primarily during cytokinesis but not during metaphase (Kanfer et al., 2015). CENP-F interacts with EB1, a protein that associates with the growing tip of microtubules. The Miro/CENP-F/EB1 interaction propels mitochondria movement towards the cleavage furrow. As astral microtubules grow towards the midzone during anaphase, mitochondria attach to the tips and are forced into the proper location. In support of this
hypothesis, it was seen that mitochondrial localization at the furrow was disrupted by treatment of the microtubule stabilizing drug taxol (Lawrence and Mandato, 2013b). EB1 is known to be associated with dynamic microtubules, and this interaction is disrupted when microtubules are stabilized especially in the case of taxol treatment (Shannon et al., 2005). As such, taxol would displace EB1 from the growing tips of microtubules preventing the mitochondria from hitching a ride towards the cleavage furrow through the EB1/CENP-F/Miro complex. Mitochondrial movement towards the contractile ring also depends on kinesin driven motility via astral microtubules. When motorless KIF5B or Miro without the transmembrane domain is expressed, mitochondrial movement towards the midzone markedly decreases (Lawrence et al., 2016). Likely a combination of kinesin-driven movement and EB1/CENP-F work in concert to transport mitochondria to the cleavage furrow.

From these studies, there is substantial evidence to believe that mitochondrial inheritance has an active component to inheritance even though mitochondria tend to fall into the category of passive inheritance. Regardless of inheritance type, the positioning of the mitochondria would also be important for the inheritance process. Yet, it is still not known how mitochondria are initially positioned. During the work of this thesis, we have found that mitochondria are released from their attachments and position passively. If mitochondria are positioned incorrectly prior to mitosis, the mitochondria are unable to reposition themselves and can be inherited asymmetrically. Last, we found that regulation of mitochondrial positioning during mitosis is ultimately important for inheritance and fidelity of cell division.
Chapter 2: Phosphorylation-Induced Motor Shedding is required at Mitosis for Proper Distribution and Passive Inheritance of Mitochondria

This chapter is a modified version of the manuscript entitled “Phosphorylation-Induced Motor Shedding is required at Mitosis for Proper Distribution and Passive Inheritance of Mitochondria” with Jarom Y. Chung, Judith A. Steen, and Thomas L. Schwarz. (Cell Reports, in preparation)
Summary

Interphase mitochondria associate with microtubules, but mitotic mitochondria dissociate from both spindle and astral microtubules and instead localize in the cell periphery. This redistribution is mediated not by active transport or tethering of the mitochondria; rather it is caused by the dissociation of the motor proteins kinesin, dynein, and dynactin from their mitochondrial adaptors, Miro and Milton. The motors that otherwise link the mitochondria to microtubules are thereby shed from the mitochondrial surface. The shedding is driven by phosphorylation of targets both on mitochondria and in the cytoplasm by CDK1 and Aurora A. Overriding the shedding and recruiting motors to mitotic mitochondria alters their localization, prevents their proper symmetrical distribution, and disrupts the balanced inheritance of mitochondria to daughter cells. Moreover, if mitochondria decorate the spindle apparatus due to dynein recruitment onto mitotic mitochondria, their presence on the spindle arrests cell cycle progression and produces binucleate cells. Thus the regulated release of motors from the mitochondrial surface is a critical mitotic event.

Introduction

Preceding cell division, the cell orchestrates processes that will ensure two complete daughter cells. While many investigations focus on chromosomal duplication and segregation, recent studies have shown that mitochondria are also affected: they increase their number during the early stages of mitosis through biogenesis and fission (Kashatus et al., 2011; Martinez-Diez et al., 2006). Mitochondria perform vital roles including producing cellular ATP, maintaining calcium homeostasis, and regulating cell survival. Because they contain their own DNA, they cannot be formed de novo and because their functions are critical at all cell stages, their proper
inheritance needs to be ensured among daughter cells. In yeast, mitochondrial inheritance depends on movement of healthy mitochondria along actin filaments into the budding daughter cell (Westermann, 2014). Unlike in yeast, metazoan mitochondria primarily move along polymerized microtubules. Although much is known about mitochondrial inheritance in yeast, the mechanism in metazoans and the role of microtubules in mitochondrial inheritance remains unknown (Mishra and Chan, 2014).

Two types of organelle inheritance are hypothesized for mitosis: active and passive. Active segregation is carried out through attachment to microtubules primarily, a prime example being chromosomal inheritance. For passive segregation, it is thought that an increased number of particles allows for stochastic and balanced inheritance into daughter cells (Symens et al., 2012). As such, mitochondria are thought to undergo passive inheritance, although it remains unclear how the cell ensures passive inheritance and if passive inheritance is in fact occurring. As the cell enters mitosis, mitochondria fragment due to increased activity of DRP1, the mitochondrial fission factor, and degradation of the mitochondrial fusion factor MFN1 by MARCH-V (Kashatus et al., 2011; Park and Cho, 2012). The increased number of mitochondria is thought to allow for their even distribution and the subsequent likelihood of equal inheritance. However, the passive inheritance hypothesis also depends on release from tethering elements and the even distribution of mitochondria throughout the cytoplasm, which fragmentation alone does not guarantee (Mishra and Chan, 2014). Conflicting reports have implicated microtubules and actin as active drivers of mitochondrial inheritance, but it remains unknown how these cytoskeletal elements would position mitochondria during cell division (Lawrence and Mandato, 2013b; Lee et al., 2007; Rohn et al., 2014). One study observed that mitotic mitochondria
associate with the + ends of microtubules via Cenp-F, a protein previously known to function at kinetochores (Kanfer et al., 2015).

Mitochondria move along microtubules by means of a motor adaptor complex consisting of the atypical RHO-GTPase Miro (RHOT1/2) that resides on the outer mitochondrial membrane, the motor adaptor Milton (TRAK1/2, OIP106/98), kinesin heavy chain (KHC, KIF5), and dynein/dynactin complexes. Both Miro and Milton are essential for attaching the motor proteins to the mitochondrial surface and hence for mitochondrial movement (Fransson et al., 2006; Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002; van Spronsen et al., 2013). Additionally, mitochondria are known to attach to actin and the ER for anchoring, calcium buffering, and fission (de Brito and Scorrano, 2008; Pathak et al., 2010). These attachments contribute to proper mitochondrial distribution and function throughout the cell during interphase.

To date, it is still unclear how mitochondrial distribution changes during mitosis and if these cytoskeletal associations or the motor adaptor complex affect distribution and subsequent inheritance (Mishra and Chan, 2014). While examining mitotic cells, we observed a marked shift in the relationship of mitochondria to the microtubule network. We tested likely mechanisms for active control of mitochondrial distribution, including peripheral tethers. We found evidence instead for passive mitochondrial inheritance. We therefore examined the regulation of the motor adaptor complex and demonstrated that release of motors from the complex is required for proper mitochondrial inheritance and the fidelity of cell division.
Results

Mitochondria are released from microtubules during cell division and remain peripheral to the mitotic spindle.

We have examined by confocal microscopy the relationship of mitochondria to the microtubule network in HeLa cells that were synchronized with a double thymidine block (Figure 2.1). During interphase, the mitochondria are overwhelmingly associated with microtubules, as expected from their known dynamic movement along these tracks (Ball and Singer, 1982). In contrast, mitochondria do not colocalize with the mitotic spindle, a phenomenon most apparent in an optical section through the center of the cell. In fixed images and live imaging, mitochondria appear to be released from microtubules once the cell enters mitosis and indeed appear to be repelled from the space occupied by the spindle (Figure 2.1). When viewed in a 3D reconstruction, mitotic cells have a central core of the spindle and chromosomes, which is surrounded by a peripheral mitochondrial zone that does not appear to have contact with even the outermost shell of spindle microtubules. Even in the periphery of the cell, where mitochondria can be close to astral microtubules, the mitochondria are not oriented adjacent or parallel to these microtubules, as they are in interphase, but rather appear to ignore the microtubules in their vicinity. We calculated percent overlap between mitochondria and tubulin as a fraction of the total mitochondrial signal during interphase versus mitosis and found that overlap significantly decreases during mitosis (Figure 2.2). The residual overlap probably represents the incidental overlap of astral microtubules and peripheral mitochondria. This was also observed in HEK293T cells and COS cells and in primary cultures of rat embryonic fibroblasts (data not shown). Mitochondria thus appear to detach from microtubules during cell division.
**Figure 2.1**

Interphase and mitotic HeLa cells were stained for TOM20, mitochondrial marker (magenta), tubulin (green), and DNA (Hoechst 33342, blue) and imaged by confocal microscopy at 63x and 100x. Scale bars represent 5 microns.

**Figure 2.2**

Percent of the total mitochondrial area that overlapped with tubulin at interphase, prophase, prometaphase, metaphase, and anaphase. Data are represented as mean ± SEM. Scale bars represent 5 microns.
To characterize further the timing of when mitochondria detach from microtubules, we analyzed both live and fixed cells during stages of mitosis (Figure 2.4). We graphed the distribution of DNA, tubulin, and mitochondria during metaphase using the “Radial Profile” ImageJ plugin (Baggethun, 2009). The center of the DNA signal was used as the center reference point (Figure 2.3) and the average fluorescence intensity per area was calculated for concentric circles emanating from this center. The values at each distance were averaged from 30 cells to create a radial distribution plot (Figure 2.4). During interphase, tubulin and mitochondria have similar distribution patterns, which persists as the cell enters prophase. However, there is a dramatic change when the cell enters prometaphase. As seen by live imaging (data not shown), the microtubule network depolymerized during prometaphase and shifted towards the center of the cell in order to form the mitotic spindle. In contrast, the mitochondria remained in place without any consistent translocation indicative of microtubule-based movement towards the periphery. The mitotic cell, however, became rounded and in consequence the cell perimeter moved closer to where the mitochondrial already were present. Thus the largely stationary mitochondria appeared more peripheral while the spindle formed in the mitochondria-free zone that was formerly the nucleus. We noticed that the mitochondria always maintained a slight distance from the forming spindle during prometaphase and appeared to be excluded from a space surrounding the spindle that contained dynamic microtubules. This may be due either to the recently reported mechanism by which mitochondria ride on the + ends of mitotic microtubules (Kanfer et al., 2015) or to simple steric occlusion by the microtubules.
Figure 2.3 Schematic showing how the radial distributions of tubulin and mitochondria were calculated using the center of the spindle as the center reference point. Figure 2.4 The radial distributions of tubulin and mitochondria were determined for 30 mitotic cells and averaged for each of the mitotic phases. Images are representative of the phase. Scale bars represent 5 microns.
Mitochondria remained dissociated from microtubules during metaphase until they reattached during anaphase (Figure 2.4). This observation was confirmed by measuring the percent of mitochondrial signal that overlaps with tubulin (Figure 2.2). Overlap steadily decreased as the cell entered mitosis with a minimum of overlap of tubulin and mitochondria during metaphase. Overall, we concluded that the peripheral localization of mitochondria during mitosis is caused primarily by their release from the reorganizing microtubules and not by motor-based transport.

**Figure 2.5**

Figure 2.5 Schematic indicating how the asymmetric index at metaphase was calculated with the equation shown. **Figure 2.6** Asymmetric indices of mitochondria and tubulin were calculated for 30 metaphase HeLa cells. Data are represented as median with the interquartile range. **Figure 2.7** HeLa cells in telophase were stained for mitochondria, tubulin, and DNA. **Figure 2.8** Asymmetric indices of mitochondria and tubulin in 30 telophase cells. Data are represented as median with the interquartile range. Scale bars represent 5 microns.
To further characterize metaphase cells, we calculated the asymmetric index of the mitochondrial signal by creating a 3D projection of images that were taken as a z-stack with a spacing of 0.55 microns. The cell was divided in half along the metaphase plate, and the mitochondrial signal was measured for either half of the cell (Figure 2.5). The asymmetric index was defined as the absolute value of the difference between the two halves divided by their sum. Perfect symmetry yields a value of 0 and complete asymmetry yields 1. Wild type HeLa cells had a median asymmetry index of 0.136, which indicates on average a 13.6% fold difference between the two hemispheres (Figure 2.6). As a point of reference, the tubulin signal was more symmetric with a median index of 0.076 (Figure 2.6). A similar analysis of HeLa cells during telophase right before cytokinesis yielded an asymmetric index for mitochondrial signal of 0.120, similar to that at metaphase (Figure 2.7, 2.8).

**Mitochondria are passively localized away from the spindle.**

One potential mechanism for the redistribution of mitochondria during mitosis would be their tethering to a cytoskeletal element that held them away from the spindle. We therefore used nocodazole to depolymerize microtubules. As seen by live cell microscopy (Figure 2.9), as the microtubule-rich spindle depolymerized, the mitochondria immediately moved to occupy that space and were only excluded from the area occupied by the dense chromosomes (Figure 2.10). These data suggest that mitochondria are not tethered to elements in the periphery. To further examine this possibility, we tested the effects of two known tethers for mitochondria.

Mitochondria can interact with actin microfilaments (Pathak et al., 2010) and it has been suggested that their peripheral localization arises from association with the cortical actin network (Lee et al., 2007). To test this hypothesis, we synchronized HeLa cells and treated with either
DMSO (the vehicle control) or Latrunculin A, a depolymerizing agent, for ten minutes prior to fixation. In control conditions, however, the actin-rich region, as visualized with phalloidin staining, and the mitochondrial zone were quite distinct. Actin was at the extreme periphery of the cell and the mitochondria resided between that actin shell and the spindle (Figure 2.11). Moreover, Latrunculin treatment abolished the actin network but the mitochondrial distribution was not substantially altered. The mitochondria did not collapse onto the mitotic spindle; instead, mitochondria remained in the periphery similar to the control (Figure 2.12).

**Figure 2.9**

**Figure 2.10**

*Figure 2.9* HeLa cells were transfected with Mito-dsRed and GFP-tubulin and synchronized with a double thymidine block. Following thymidine release, cells were treated with nocodazole (0 min) and images were taken every 2 minutes. *Figure 2.10* HeLa cells were synchronized in mitosis and were treated with either DMSO (Control) or Nocodazole for 10 minutes prior to fixation. Cells were imaged and stained for tubulin, mitochondria, and DNA. Scale bars represent 5 microns.
The percent overlap of mitochondria and tubulin decreased in the Latrunculin treatment compared to DMSO, perhaps due a decrease in astral microtubules. Latrunculin treatment also did not appear to change significantly the asymmetry index of mitochondria during metaphase.

**Figure 2.11**

Composite | Tubulin | Mitochondria | Actin
---|---|---|---
DMSO | | |
Latrunculin A | | |

**Figure 2.12**

**Figure 2.13**

**Figure 2.11** HeLa cells were synchronized by a double thymidine block to obtain metaphase cells and then treated for 10 min with either DMSO or Latrunculin A prior to fixation. Mitochondria (anti-TOM20, magenta), tubulin (green), and actin filaments (white) were immunostained and imaged by confocal microscopy. **Figure 2.12** The averaged radial distributions of 30 mitotic cells treated with either DMSO or Latrunculin A. **Figure 2.13** The percent overlap of the mitochondrial and tubulin signals of DMSO and Latrunculin A treated cells. Data are represented as mean ± SEM. Scale bars represent 5 microns.
Figure 2.14 HeLa cells were transiently transfected with STIM1 WT or STIM 10A. Immunostaining for STIM1 (white) identified transfected cells and confirmed that the 10A mutation prevented ER-dissociation from microtubules. After synchronization, the radial distributions of tubulin and mitochondria Figure 2.15 and percent mitochondrial overlap with tubulin Figure 2.16 were determined for 30 cells. Data are represented as mean ± SEM. Scale bars represent 5 microns. Figure 2.17 HeLa cells were transiently transfected with STIM1 WT or STIM 10A in conjunction with the ER marker RFP-Sec61B. Cells were synchronized in mitosis and imaged by confocal microscopy. Cells were also stained for tubulin and DNA. Mitochondria are unaffected by changes to mitotic endoplasmic reticulum.
Mitochondria are also known to be associated with the endoplasmic reticulum (ER) (de Brito and Scorrano, 2008). The ER, like mitochondria, associates with microtubules during interphase, but is released from the microtubules during mitosis via the phosphorylation of STIM1 (Smyth et al., 2012). We therefore tested the possibility that the redistribution of mitochondria arises because their close association with the ER causes them to follow the ER as it comes off the spindle. To test the effect of ER attachment on mitochondria during cell division, we employed a phosphoresistant STIM1 (10A) that mislocalizes ER onto the spindle (Smyth et al., 2012) (Figure 2.17). We transiently expressed either STIM1 wild type (WT) or the mutant version (10A) in HeLa cells, followed by synchronization and imaging (Figure 2.14). Although the STIM1 10A construct forced ER onto the spindle, it failed to alter mitochondrial localization. The spindle in the STIM1 10A condition appeared to be slightly larger and the mitochondria appeared to have been pushed further into the periphery (Figure 2.15) and thus, rather than bringing mitochondria onto the spindle, there was a decrease in the extent of mitochondria/microtubule overlap (Figure 2.16).

Motor proteins release from mitochondria during cell division.

The switch from an intimate relationship of mitochondria and microtubules in interphase to their abrupt divorce during mitosis suggested a change in the proteins that underlie the relationship rather than tethering to another element. The clearest connection of the organelle and the microtubules is that mediated by the motor proteins dynein, dynactin, and kinesin. We therefore asked if the motor proteins were degraded or lost either their association with mitochondria or their ability to interact with mitotic microtubules. Kinesin and dynein interact
Figure 2.18 HeLa cells were synchronized into interphase (by thymidine block) or mitosis (nocodazole-induced arrest). Whole cell lysates (WCE) and isolated mitochondria (Mito) were probed for each of the motor adaptor complex proteins. Levels of the motor protein subunits, but not the adaptors Milton and Miro, were reduced on mitotic mitochondria. The bands for DIC, p150, and Milton also migrate differently during mitosis. Anti-CyclinB is shown to verify the phase of the mitotic fraction and the mitochondrial protein ATP5b was probed as a control for equal mitochondrial content. Figure 2.19 Myc-hMilton1 was immunoprecipitated from lysates of interphase or mitotic HeLa cells and then incubated with calf intestinal phosphatase (CIP) either with the phosphatase inhibitor NaVO₄ (-CIP) or without the inhibitor (+CIP). Phosphatase treatment reversed the band-shift for mitotic Milton.

with mitochondria via the motor adaptor complex of Milton and Miro. HeLa cells were synchronized using a double thymidine block for interphase (G1/S phase) and were synchronized with a single thymidine block followed by addition of nocodazole for mitotic synchronization. Cells were lysed and analyzed by western blot and cyclin B levels were determined to confirm that cells were in mitosis. Because dynein and dynactin are composed of many components, we
measured dynein intermediate chain (DIC) to assay dynein and p150 to assay dynactin. Levels of DIC, conventional kinesin-1 (KHC), p150, Milton, and Miro did not significantly change between interphase and mitosis (Figure 2.18). DIC and p150 both exhibited a band shift, which is known to be due to phosphorylation. Milton also exhibited an apparent molecular weight shift. When immunoprecipitates of overexpressed myc-hMilton1 from interphase or mitotic cells were treated with active phosphatase (+CIP), the mitotic band shift of Milton was reversed indicating that Milton also undergoes phosphorylation during mitosis (Figure 2.19).

**Figure 2.20**

![Image of cell microscopy](image)

**Figure 2.20** HeLa cells transiently expressed myc-hMilton1 (Milton) and either (A) KIF5C-CFP (KHC), (B) DIC2C-RFP (DIC), (C) pEGFPC2-p150 (p150), or (D) HA-Miro1 (Miro). Cells were imaged during interphase and mitosis by confocal microscopy. During mitosis, each of the motor proteins is present on the spindle microtubules. For KHC, the redistribution is most apparent because in interphase it is recruited almost exclusively to mitochondria and moves the mitochondria to aggregates in the periphery of the cell. In mitosis, KHC is diffuse in the cytosol and on the spindle. Scale bars represent 5 microns.
To determine if the complex remained on mitochondria, mitochondria isolated from cells in interphase and mitosis were examined. DIC, p150, and KHC levels on mitochondria decreased during mitosis (Figure 2.18), but Miro and Milton remained associated with the mitochondria and the mitochondrial Milton exhibited the phosphorylation-associated band shift during mitosis. Cyclin B also associates with mitochondria, as has been previously reported (Kashatus et al., 2011). The selective loss of the motor proteins from mitotic mitochondria was also observed when S-trityl-L-cysteine was used instead of nocodazole to synchronize cells (data not shown). Myc-hMilton1 was expressed with HA-Miro1 and components of the motors in HeLa cells for analysis by immunocytochemistry at interphase and metaphase. Miro and Milton colocalized with the mitochondrial marker TOM20 both during interphase and mitosis and did not overlap with the spindle (Figure 2.20). As previously observed (Glater et al., 2006), overexpressed KHC colocalized with Milton and mitochondria during interphase and induced peripheral aggregates of mitochondria, consistent with excessive transport to (+) ends. During mitosis, KHC was enriched on the spindle but not on mitochondria, consistent with the biochemical observation of kinesin loss from mitochondria (Figure 2.20). Similarly, DIC overexpression with Milton and Miro caused perinuclear clustering of mitochondria during interphase, consistent with excess traffic to (−) ends, although DIC was not as exclusively mitochondrial as KHC. During mitosis, overexpressed DIC was diffuse in the cytosol but also very noticeably present on the spindle. p150, when overexpressed with Milton and Miro was diffusely cytoplasmic during interphase, but highly enriched on the mitotic spindle during cell division. The ability of KHC, DIC and p150 to associate with the mitotic spindle excludes a model in which mitochondrial dissociation from microtubules is due to the loss of the motors’ affinity for mitotic microtubules. Rather, the biochemical and immunocytochemical characterization, pointed to a motor-shedding hypothesis
in which Miro and Milton remain on mitochondria but the motor protein complexes of dynein, dynactin, and kinesin detach from these adaptor proteins during cell division.

**Motor release is inducible by phosphorylation and reversible.**

Because Milton phosphorylation could not explain the mitotic mitochondrial behavior, we broadened our focus to determine whether the ability of motors to bind to mitochondria was more dependent on the state of the organelle or the state of the cytosol. In order to create *in vitro* motor shedding, we fractionated interphase and mitotic cells to obtain mitochondrial and cytosolic fractions. Mitochondria from each phase were then mixed with either interphase or mitotic cytosol. After a two-hour incubation, mitochondria were isolated again and analyzed by western blot (Figure 2.21). Interphase mitochondria, which originally had high levels of both DIC and KHC, lost these motors when incubated with mitotic cytosol as compared to interphase cytosol (Figure 2.22). We did not, however, observe the large phosphorylation-dependent band shift in Milton or DIC, indicating that the *in vitro* incubation did not fully recapitulate all the events that occur in intact cells. In the reciprocal experiment, mitotic mitochondria were incubated with either interphase or mitotic cytosol. DIC and KHC reattached following incubation with interphase cytosol compared with mitotic cytosol conditions (Figure 2.22). We concluded that the state of the cytosol was key in determining motor attachment or detachment: mitotic cytosolic factors were sufficient to release motors from mitochondria, and interphase cytosolic factors were able to reattach motors.

Because phosphorylation governs much of mitosis, we hypothesized that phosphorylation triggered motor shedding. CIP was therefore used to dephosphorylate either mitochondrial or cytosolic fractions isolated from mitotic cells to determine if it could induce motors to reattach
Schematic (Figure 2.21) and immunoblot (Figure 2.22) of a biochemical assay to determine if interphase or mitotic cytosol can either reverse or induce motor release. Mitochondria were isolated from either interphase (I) or mitotic (M) cells and incubated with either interphase or mitotic cytosol. Mitochondria were then re-isolated and assayed for the presence of the indicated proteins. Mitotic cytosol could induce loss of motors from interphase mitochondria and interphase cytosol could induce their reattachment to mitotic mitochondria.

(Figure 2.23). When untreated mitotic mitochondria were incubated with untreated mitotic cytosol, motors, as expected, did not significantly reattach to mitochondria (Figure 2.24). Combining CIP-treated mitochondria with untreated cytosol caused a slight increase in bound DIC and KHC. When CIP-treated cytosol was added to untreated mitochondria, KHC associated significantly more with the mitochondrial fraction compared to the previous two conditions, but DIC levels did not change significantly. Mitochondrial DIC increased robustly only when both mitochondria and cytosol had been treated with CIP. Thus motor shedding is dependent on phosphorylation and dynein detachment is driven by changes to both cytosolic and mitochondrial components while the association of KHC with mitochondria is primarily dependent on cytosolic phosphorylation.
Figure 2.23-2.24 Schematic (Figure 2.23) and immunoblot (Figure 2.24) of an assay to determine whether calf intestinal phosphatase (CIP) treatment of either a mitochondrial or cytosolic fraction could allow motors to reattach to mitochondria. The separate fractions were treated with either CIP alone (+) or CIP and the phosphatase inhibitor NaVO₄ (-) as indicated and then recombined. Mitochondria were isolated and immunoprobed for the indicated proteins.

**Milton and Kinesin phosphorylations are not sufficient to cause microtubule release.**

The large shift in the gel migration of Milton during mitosis suggested that Milton phosphorylation might cause the motors to detach. Mass spectroscopy identified 22 serine/threonine phosphorylation sites on myc-hMilton1 in mitotic HeLa cells and 4 sites present in both interphase and mitotic cells. Sequence analysis of human Milton1 for potential CDK1 sites containing the loose consensus sequence (S/T-P) identified additional potential sites. We therefore synthesized a mutant hMilton1 with 28 phosphoresistant alanine mutations (Figure 2.25) which, when expressed in HeLa cells, prevented the gel migration shift during mitosis (Figure 2.26). Despite the mutations, the Milton 28A mutant colocalized with mitochondria and did not differ from wild type Milton in the levels of motor proteins on interphase mitochondria (Figure 2.27). However, DIC and KHC levels on mitochondria also decreased equivalently.
Figure 2.25 Schematic of the 28 sites on human Milton1 that were mutated to alanine to prevent phosphorylation. Sites were selected by a compilation of mass spectrometry of Milton immunoprecipitated from mitotic and interphase HeLa cells and from sites (red) with sequences that match the CDK1 target consensus S/T-P (black). Figure 2.26 Mitochondria were isolated from cells expressing either myc-hMilton1 wild type (WT) or myc-hMilton1 with 28 alanine substitutions (28A) during interphase (I) or mitosis (M) and immunoprobred for the indicated proteins. The 28A mutation prevented the large shift in migration of Milton during mitosis, but did not prevent the shedding of the motor proteins. Figure 2.27 Cells expressing HA-Miro1, GFP-Tubulin, and either myc-hMilton1 WT or 28A were immunostained for the mitochondrial marker TOM-20, Milton, and tubulin. The 28A mutation did not alter mitochondrial distribution during mitosis.

Figure 2.25 Schematic of the 28 sites on human Milton1 that were mutated to alanine to prevent phosphorylation. Sites were selected by a compilation of mass spectrometry of Milton immunoprecipitated from mitotic and interphase HeLa cells and from sites (red) with sequences that match the CDK1 target consensus S/T-P (black). Figure 2.26 Mitochondria were isolated from cells expressing either myc-hMilton1 wild type (WT) or myc-hMilton1 with 28 alanine substitutions (28A) during interphase (I) or mitosis (M) and immunoprobred for the indicated proteins. The 28A mutation prevented the large shift in migration of Milton during mitosis, but did not prevent the shedding of the motor proteins. Figure 2.27 Cells expressing HA-Miro1, GFP-Tubulin, and either myc-hMilton1 WT or 28A were immunostained for the mitochondrial marker TOM-20, Milton, and tubulin. The 28A mutation did not alter mitochondrial distribution during mitosis.

during mitosis and mitochondria in cells expressing the 28A mutant were in the periphery and appeared dissociated from the microtubules despite the absence of the phosphorylation sites (Figure 2.26). Were the phosphorylations necessary for dissociation of the motor proteins or the peripheral localization of mitochondria, the 28A mutant would be predicted to have a dominant
effect over any endogenous wild type Milton. We concluded therefore that the phosphorylation of these sites was not required for mitochondrial redistribution or motor shedding during cell division.

**Figure 2.28**

![Schematic and immunoblot of an assay to determine the significance of Milton phosphorylation for motor shedding during mitosis.](image)

**Figure 2.29**

<table>
<thead>
<tr>
<th>Mitotic Cytosol ± CIP</th>
<th>Myc-Milton Construct</th>
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<td>- - + +</td>
<td>WT 28A WT 28A</td>
</tr>
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Since DIC shedding was in part dependent on phosphorylation of mitochondrial proteins, we examined the contribution of Milton phosphorylation to the mitochondrial component of that event. We overexpressed the 28A mutant and wild type constructs, isolated mitotic mitochondria, and combined them with cytosol pretreated either with CIP or inactive CIP (Figure 2.28). Mitotic mitochondria incubated with mitotic cytosol treated with inactive CIP did not have any DIC reattach as expected. When incubated with cytosol treated with active CIP, the 28A-expressing mitochondria did not have an increase of DIC attachment compared to the WT-expressing mitochondria (Figure 2.29). We concluded that Milton phosphorylation alone was not responsible for DIC loss; it likely involves phosphorylation of other mitochondrial proteins.
Given that Kinesin detachment was driven primarily by phosphorylation in the cytosolic fraction, we tested if phosphorylation on Kinesin itself drove release. Proteomic studies of HeLa cells during the cell cycle identified two conserved mitotic phosphorylation sites (S917, S938) on kinesin heavy chain (Olsen et al., 2010) that fall near the Milton-binding domain (residues 810-891) (Glater et al., 2006). We created a phosho-resistant construct and saw neither increased Kinesin attachment during mitosis (Figure 2.30) nor a change in mitochondrial distribution (Figure 2.31). We concluded that preventing phosphorylation at those sites was not sufficient to prevent motor shedding.

**Figure 2.30**

<table>
<thead>
<tr>
<th>Mitochondrial Phase</th>
<th>KHC WT</th>
<th>KHC 917/933A</th>
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<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
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</table>

**Figure 2.31**

G. Mitochondria were isolated from cells expressing either KIF5C-CFP wild type (WT) or KIF5C-CFP with 2 alanine substitutions during interphase (I) or mitosis (M) and immunoprobed for the indicated proteins. The KIF5C-CFP mutations did not prevent the shedding of kinesin. H. Cells expressing either KIF5C-CFP wild type or KIF5C-CFP 917/933A were immunostained for the mitochondrial marker TOM-20, KIF5C-CFP, and tubulin. The KIF5C mutations did not alter mitochondrial distribution during mitosis.

**CDK1 causes Dynein release, and Aurora A causes Kinesin release**

Cyclin-dependent kinase 1 (CDK1) and its cofactor cyclin B are the primary drivers of mitosis; we therefore tested whether CDK1 could induce motor shedding when applied to interphase mitochondria. Isolated interphase mitochondria were treated with active CDK1 for an hour and then washed (Figure 2.32). DIC levels on the CDK1-treated mitochondria decreased.
compared to those untreated (Figure 2.33). We concluded that purified CDK1 was sufficient for DIC shedding. In contrast, there wasn’t a significant change in KHC binding. CDK1 may still be an important upstream trigger but, for kinesin detachment, it did not appear to suffice in the absence of other cytosolic factors. Therefore we tested other downstream kinases and found that Aurora A was sufficient to induce kinesin shedding whereas PLK1 was not (Figure 2.33, data not shown). We found no synergistic increase of shedding when combining CDK1 and Aurora A; the two motors appear to be released by distinct mechanisms.

![Figure 2.32](image1)

![Figure 2.33](image2)

**Figure 2.32-2.33** Schematic and immunoblot of interphase mitochondria treated either with or without active purified CDK1 and/or Aurora A, reisolated, and immunoprobed for the indicated proteins.

To test the necessity of CDK1 and Aurora A in motor shedding, we synchronized cells in mitosis with nocodazole and treated with the vehicle, a CDK1 inhibitor (RO-3306), an Aurora A inhibitor (ZM 447439), or a combination of the two (Figure 2.34). As expected CDK1 inhibition reattached dynein back onto the mitochondrial surface. Additionally, kinesin also reattached
suggesting that CDK1 is upstream of kinesin release. Aurora A inhibition, however, did not increase DIC or kinesin attachment. Taken together with our in vitro data, we concluded that Aurora A was sufficient to release kinesin but that there were other factors that could cause kinesin shedding as well. To test this hypothesis, we expressed Aurora A constitutively active (T288D) and kinase dead (K162R) in HEK293T cells synchronized in interphase (Figure 2.35). We found that the constitutively active Aurora A was sufficient to detach kinesin but not DIC, as had been observed in vitro. In addition, we found that active Aurora A shifted the gel migration of Milton, which was also detectable by in vitro reactions.

**Table 2.34**

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria</th>
<th>WCE</th>
<th>Mito</th>
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<tbody>
<tr>
<td>RO-3306</td>
<td>- + - +</td>
<td>K162R</td>
<td>K162R</td>
</tr>
<tr>
<td>ZM 447439</td>
<td>- - + +</td>
<td>T288D</td>
<td>T288D</td>
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<td>DIC</td>
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<td>KHC</td>
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<td>Milton</td>
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<td>Milton</td>
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</tr>
<tr>
<td>ATP5b</td>
<td></td>
<td>ATP5b</td>
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<td>HA-Aurora A</td>
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<td>HA-Aurora A</td>
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**Figure 2.34** Mitotic HeLa cells that were synchronized with nocodazole were treated with the CDK1 inhibitor RO-3306 and/or the Aurora inhibitor ZM447439. Isolated mitochondria were probed for the indicated proteins. **Figure 2.35** Unsynchronized HeLa cells were transfected with dominant negative Aurora A kinase (K162R) or constitutively active Aurora A kinase (T288D). Whole cell extract and mitochondria were probed for the indicated proteins.
Figure 2.36 A-B. Schematic and representative images of the rapalog system used to target an FKBP domain (Tom20-mCherry-FKBP) to mitochondria and thereby recruit either a chimeric kinesin motor (HA-KIF5B-FRB) or the dynein adaptor BICD (HA-BICD2-FRB) to mitochondria upon rapalog addition. Cells were treated with ethanol (control) or the heterodimerizer rapalog 10 minutes prior to fixation and imaged by confocal microscopy.

C. Schematic of chimeric proteins of BICD and the KIF5C motor domain that were directly tethered to the outer mitochondrial membrane. HeLa cells transiently expressing GFP-Mito or GFP-BICD2-Mito (grey) were synchronized and imaged during metaphase. Cells were immunostained and pseudocolored for the mitochondrial marker TOM-20 (magenta) and tubulin (green). BICD, but not the GFP control, induced mitochondria to cluster near the spindle poles.

Motor attachment mislocalizes mitotic mitochondria.

To determine whether motor shedding was necessary for proper mitochondrial localization during mitosis, we used two strategies for overriding the normal detachment of the motors. One approach used a temporally controlled attachment via application of the heterodimerizing drug rapalog (A/C Heterodimerizer, Clontech), which creates a link between
proteins containing the FKBP and FRB domains (Figure 2.36). The second strategy used constitutive anchoring of the motor through fusion with a mitochondrial sequence (Figure 2.38). To induce dynein attachment with rapalog, we transfected HeLa cells with a mitochondrial targeted FKBP domain (TOM20-mCherry-FKBP) and with an FRB domain attached to Bicaudal D2 (HA-BICD2-FRB), a known adaptor for dynein and dynactin (Hoogenraad et al., 2003). In interphase cells, upon addition of rapalog, the mitochondrial network collapsed onto the microtubule-organizing center (Figure 2.37), as expected from excessive dynein-mediated movement. Cells were synchronized into mitosis with a double thymidine block and were treated ten minutes prior to fixation with rapalog or ethanol, the vehicle control (Figure 2.40). Metaphase cells have two microtubule organizing centers contained at the two poles of the spindle. Upon addition of rapalog, mitochondria were no longer in the periphery and instead localized onto the spindle (Figure 2.41). Consequently, the percent overlap between mitochondria and tubulin significantly increased compared to the vehicle control (Figure 2.42). When BICD was targeted directly to the outer mitochondrial membrane (Figure 2.38), mitochondria also localized to the spindle apparatus (Figure 2.39). Thus dynein recruitment to the mitochondrial surface during cell division results in reattachment of the mitochondria to microtubules and their mislocalization onto the mitotic spindle. In addition to illustrating the necessity of dynein-shedding for proper mitochondrial relocalization, it demonstrates that dynein-microtubule interactions are not inhibited during mitosis.

We tested the effects of kinesin attachment with similar protocols. For constitutive attachment of kinesin, we created a chimeric mitochondria-targeted kinesin by fusing the KIF5C motor domain with td-Tomato and the c-terminal transmembrane domain of Miro. In interphase cells, the construct shifted mitochondria to the cell’s periphery, but also caused swollen,
**Figure 2.40** HeLa cells transiently expressing Tom20-mCherry-FKBP and the dynein-binding construct HA-BICD2-FRB were synchronized and treated with ethanol (control) or the heterodimer rapalog 10 minutes prior to fixation. Rapalog addition caused mitochondria (mCherry) to mislocalize to the spindle. **Figure 2.41-2.42** Averaged radial distribution and percent overlap of mitochondrial and tubulin signals for 30 vehicle and rapalog treated cells as in Figure 2.40. Data are represented as mean ± SEM. **Figure 2.43** HeLa cells transiently expressing Tom20-mCherry-FKBP and HA-KIF5B MD-FRB were synchronized and treated with either ethanol (control) or rapalog for 10 minutes prior to fixation. Rapalog caused mitochondria to assume a more peripheral localization. **Figure 2.44-2.45** Averaged radial distribution and percent overlap of mitochondrial and tubulin signals for 30 vehicle and rapalog treated cells. Data are represented as mean ± SEM. Scale bars represent 5 microns.
circularized mitochondria. The rapalog strategy provided a cleaner system for the analysis: the KIF5B motor domain was fused with an FRB domain (HA-KIF5B MD-FRB) so that it could be bound via rapalog to the TOM20-mCherry-FKBP construct on the mitochondrial surface. During interphase, the addition of rapalog drove mitochondria to the periphery (Figure 2.37), as expected for the (+)-end directed motor. Upon rapalog addition during metaphase, we also observed that the mitochondria were pushed towards the periphery of the cell (Figure 2.43-2.44). As a result, there was a significant decrease of mitochondria-microtubule overlap in the rapalog condition compared to vehicle treated cells (Figure 2.45). During mitosis, microtubule plus ends are found both towards the center of the spindle (kinetochore/polar microtubules) and towards the periphery (astral microtubules). Out of 90 mitotic cells analyzed only 5 cells had any mitochondria attached to the spindle, and this only occurred when we treated cells with rapalog during prometaphase. It appears that kinesin attachment during mitosis is more likely to move mitochondria peripherally despite the concentration of plus ends near the metaphase plate. This is likely due to mitochondria primarily encountering and therefore attaching to the dynamic astral microtubules along which they would then move towards the periphery.

**Motor attachment can cause mitochondrial asymmetry during metaphase and telophase.**

Besides localizing mitochondria onto the spindle, the reattachment of motors to mitochondria produced additional changes. The symmetry of mitochondrial distribution at metaphase was disrupted when either BICD or KIF5B were reattached to mitochondria, but differed in their severity (Figure 2.46). With BICD on mitochondria, the extent to which mitochondrial distribution was asymmetric depended on the timing of rapalog addition. If BICD and dynein were attached to mitochondria prior to mitosis during G2 or during prometaphase,
mitotic mitochondria were highly asymmetric compared to the vehicle control (Figure 2.47). However, if BICD and dynein were attached during metaphase, the level of symmetry matched control cells. Mitochondria also were asymmetrically distributed upon kinesin reattachment. This effect, however, was independent of when rapalog was added (Figure 2.48).

**Figure 2.46**

<table>
<thead>
<tr>
<th>Time of Rapalog Addition</th>
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<tbody>
<tr>
<td>Control</td>
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**Figure 2.46** HeLa cells transiently expressing Tom20-mCherry-FKBP and either HA-BICD2-FRB or HA-KIF5B MD-FRB were treated with ethanol (control) or rapalog at G2, prometaphase, or metaphase and imaged by confocal microscopy at metaphase. The asymmetric index of metaphase cells was calculated on 3D projections of treated cells for BICD-FRB transfected cells. **Figure 2.47** and KIF5B-FRB transfected cells **Figure 2.48**. Scale bars represent 5 microns.
HeLa cells were transiently transfected as in Figure 2.46 and imaged after proceeding to telophase. The mitochondrial contents of the two emerging daughters cells were used to calculate an asymmetry index for cells expressing BICD-FRB (Figure 2.50) or KIF5B-FRB (Figure 2.51). Data are represented as median with the interquartile range. Scale bars represent 5 microns. Statistical comparisons are relative to control.

To determine if the asymmetric mitochondrial distribution established in metaphase would persist throughout mitosis, we allowed treated and control cells to proceed into telophase followed by fixation and imaging (Figure 2.49). Overall there was a lesser degree of asymmetry for both BICD and KIF5B conditions compared to the high asymmetry observed during metaphase (Figure 2.50). The asymmetry induced by KIF5B attachment persisted significantly into telophase (Figure 2.51), although it was also less severe than had been observed in metaphase. Further we performed live imaging of cells expressing either mitochondrial BICD or KIF5B and observed cells with an asymmetric mitochondrial distribution during metaphase as
they proceeded through mitosis. In both conditions, asymmetry that occurred in metaphase resulted in more asymmetry during telophase than control cells. Because the degree of asymmetry diminished as the cell proceeded to telophase, we concluded that compensatory mechanisms act to normalize the distribution. Like others (Lawrence and Mandato, 2013b), we saw a net movement of mitochondria towards the cleavage furrow (Figure 2.49), which may promote symmetric inheritance.

**Mitochondrial localization to the spindle interferes with progression through mitosis.**

To measure the effect of mitochondrial attachment on the duration of mitosis, we synchronized cells expressing the mito-FKBP construct and either BICD-FRB or KIF5B-FRB. Cells were released from thymidine block, treated with rapalog or the vehicle control, and beginning 8 hours later, when the maximum number of cells was in mitosis, samples were removed every 30 minutes and fixed. The percentage of mitotic cells was calculated for each time point for both vehicle and rapalog treated cells. In the absence of rapalog, or when rapalog recruited KIF5B to mitochondria, 30% of cells were mitotic 8 hours after release from thymidine, and this percentage gradually decreased over the next 11 hours to less than 10% (Figure 2.53). In contrast, BICD-FRB transfected cells treated with rapalog did not exit mitosis; the percent in mitosis after 11 hours remained at 25% (Figure 2.52).

To assess the impact of mitochondrial redistribution onto the spindle on the completion of cytokinesis, we determined whether it resulted in multinucleated cells. HeLa cells transfected with BICD-FRB and mito-FKBP were synchronized and treated with either rapalog or the vehicle control during G2. Cells were allowed to proceed through one cell division (~16 hours after thymidine release) before fixation. Binucleate cells were rare in control conditions (3/90
cells) but increased significantly to greater than 50% of rapalog treated cells (Figure 2.54-2.55). Expression of BICD that was constitutively targeted to mitochondria also significantly increased binucleate cells although to a lesser extent (Figure 2.57-2.58). In contrast, recruitment of KIF5B-FRB to mitochondria with rapalog did not significantly enhance the number of binucleate cells (Figure 2.54-2.55). Thus, while redistribution of mitochondria to the cells periphery with kinesin recruitment may cause asymmetry, it neither delayed mitotic progression nor blocked cytokinesis to create binucleate cells. In contrast, redistribution of mitochondria onto the spindle strongly interfered with mitotic progression and resulted in a high percentage of binucleate cells.

**Figure 2.52**

**Figure 2.53**

**Figure 2.52-2.53** HeLa cells transiently expressing Tom20-mCherry-FKBP and either HA-BICD2-FRB (Figure 2.52) or HA-KIF5B MD-FRB (Figure 2.53) were treated with ethanol (control) or rapalog. The percentage of transfected cells in mitosis was determined at the indicated times after release from thymidine block. Vehicle treated cells and those expressing KIF5B-FRB proceeded normally through mitosis, but those expressing BICD-FRB transfected did not complete mitosis after G2-rapalog addition. Data are represented as mean ± SEM. Scale bars represent 5 microns.
Figure 2.54 HeLa cells were transiently transfected with the following constructs: Tom20-mCherry-FKBP and HA-BICD-FRB (Mitochondria BICD); Tom20-mCherry-FKBP and HA-KIF5B motor domain-FRB (Mitochondria KIF5B); PEX-RFP-FKBP and HA-BICD-FRB (Peroxisome BICD); STIM1 WT (ER STIM1 upper panel) or STIM1 10A (ER STIM1 lower panel). The cells with FKBP and FRB constructs were treated with either ethanol (control) or rapalog (experimental; Rap) during G2. Tubulin (green), DNA (blue) and mCherry/Tom20 (mitochondria, magenta) or RFP (peroxisomes, magenta) were imaged by confocal microscopy.

Figure 2.55 The average percentage of multinucleated cells from three independent experiments were averaged and compared by Student’s t-test. Data are represented as mean ± SEM. Figure 2.56 90 cells for each condition were examined and the number of binucleated cells is indicated. Scale bars represent 5 microns.
Figure 2.57 Representative confocal images of HeLa cells transiently transfected with GFP-Mito or GFP-BICD2-Mito (grey). Cells were immunostained and pseudocolored for the mitochondrial marker TOM-20 (magenta) and tubulin (green). **Figure 2.58** The average percentage of multinucleated cells from three transfections as in (A) were averaged and compared by a student’s t-test. Data are represented as mean ± SEM. **Figure 2.59** HeLa cells were transfected with PEX-RFP-FKBP and HA-BICD2-FRB and treated with ethanol (control) or rapalog. Cells were synchronized and imaged during metaphase. BICD-induced recruitment of dynein caused peroxisomes to localize to microtubules at the spindle poles. Scale bars represent 5 microns.

The binucleate cells might have arisen either as a consequence of mitochondrial interference with the spindle or because the mislocalization of dynein onto the mitochondria.
prevented normal dynein functions during mitosis. We therefore used a peroxisome targeted FKBP domain (PEX-mRFP-FKBP) to sequester dynein onto peroxisomes and compared the consequences to those of mitochondrial recruitment. Recruitment of dynein mislocalized peroxisomes onto the spindle (Figure 2.59) but did not significantly increase binucleate cells compared to vehicle control (Figure 2.54-2.55). That bulky mitochondria but not the smaller peroxisomes were interfering with proper mitosis, though both would sequester dynein, suggested that it was not dynein localization per se, but rather the steric consequences of mitochondria on the spindle that was deleterious. To further test this hypothesis, we used STIM1 10A to prevent ER release from spindle microtubules. Cells were transfected with either STIM1 wild type (WT) or the phosphoresistant 10A mutant and after 48 hours synchronized as before and fixed to analyze the percentage of binucleate cells. Although this chronic change in ER behavior differs from the acute changes to organelles induced by rapalog addition, binucleate cells increased significantly in the STIM1 10A expressing cells (Figure 2.54-2.55). Thus, the presence on the spindle of large organelles, whether mitochondria or the ER, will interfere with mitosis and cause binucleate cells.

Discussion

We have now determined that (1) mitochondria are released from microtubules during cell division; (2) the peripheral distribution of mitochondria is not through motor-based transport or by a tethering mechanism, but rather that mitochondria passively maintain their peripheral localization established during interphase; (3) passive inheritance requires the dissociation of dynein and kinesin motors from the Miro/Milton motor adaptor complex and as a result the motors are shed from mitochondria; (4) motor detachment is due to phosphorylations by CDK1
and Aurora A kinase, with substrates both on mitochondria and in the cytoplasm; (5) when dynein loss is circumvented by forcing dynein onto the mitochondrial surface, the mitochondria will localize to the spindle apparatus, whereas kinesin reattachment forces mitochondria further into the periphery; (6) motor shedding is crucial to correct mitochondrial distribution during metaphase and balanced mitochondrial inheritance by daughter cells; and (7) if mitochondria are present on mitotic microtubules, progression through the cell cycle is arrested and binucleate cells arise.

Prior to this study, there were conflicting results about mitochondrial distribution and attachment to cytoskeletal elements like tubulin and actin (Lawrence and Mandato, 2013b; Lee et al., 2007; Martinez-Diez et al., 2006; Mishra and Chan, 2014; Rohn et al., 2014). In our study, we show that mitochondria/ microtubule interactions are disrupted as the cell enters mitosis (Figure 1). Some discrepancies may have arisen because mitochondria surrounding the microtubule-rich core appear to overlap in epifluorescent microscopy. Further we found that most mitochondria do not appear to be actively transported to the periphery, but rather that their peripheral distribution preexisted in interphase and mitochondria passively occupy the same space as the spindle forms. Although mitochondrial transport can be affected by interactions with actin and ER (de Brito and Scorrano, 2008; Pathak et al., 2010), we showed that tethering was not at play; rather, mitochondria lose all associations with actin, ER, and tubulin (Figure 2.1-2.17). In our study we focused on the mechanisms behind microtubule release given their importance to chromosomal inheritance and completion of mitosis; however, how mitochondria are released from their other interactions requires further study. Actin is important for mitochondrial inheritance in yeast (Westermann, 2014) though its role in mammalian cells has been less clear (Katajisto et al., 2015; Lawrence and Mandato, 2013b). A likely role has been
reported for actin together with myosin XIX during telophase, a stage we did not examine. This late role of actin contributes to proper mitochondrial inheritance (Rohn et al., 2014; Westermann, 2014).

Recently, and most relevant to the present study, an additional mechanism for redistributing mitochondria at mitosis has been reported in which Miro, via Cenp-F, causes mitochondria to be pushed peripherally by association with the growing plus ends of microtubules. Although we did not observe a large-scale peripheral movement of mitochondria, we did consistently notice a narrow zone surrounding the forming spindle that was clear of mitochondria. Particularly in prometaphase, this zone contained dynamic microtubules and it appears plausible that the Cenp-F-based mechanism could maintain this zone. However, we note that Cenp-F levels on mitochondria were reported to reach a minimum during this phase of mitosis (Kanfer et al., 2015). Alternatively, a delayed release of kinesin by Aurora A may contribute as well. Motor shedding and passive dissociation from microtubules, however, are likely to be the primary mechanism to keep mitochondria away from the spindle.

Release of kinesin and dynein from Miro and Milton is induced by phosphorylation. Although a previous study observed that dynein was dissociated from mitochondria (Lee et al., 2007), the state of the other components of the motor adaptor complex, kinesin, Miro, or Milton, and the physiological significance of dynein dissociation was not known. We found that Miro and Milton are maintained on the mitochondrial surface while kinesin and dynein are released preferentially (Figure 2.18-2.20). Through constitutive and temporally controlled motor attachment, we showed that motor release was necessary for disassociation from microtubules (Figure 2.40, 2.43). Even at metaphase, when the microtubules and mitochondria are most spatially distinct, dynein reattachment forced mitochondria back onto the spindle apparatus.
Further, dynein and kinesin release are both caused by phosphorylation (Figure 2.22), but they are differentially regulated (Figure 2.33-2.35) in a manner that may be important for temporal control of the dissociation. CDK1 initiates much of mitosis and CDK1-mediated dynein release may therefore precede kinesin release by the downstream actor Aurora A. In Xenopus extracts, it had been seen that addition of CDK1 also released dynein components from crude membrane fractions (Addinall et al., 2001); motor shedding may therefore be relevant for many intracellular compartments. Our data also indicated redundant phosphorylation-dependent regulation of the complex. Dynein reattachment, for instance, only occurred when both cytosolic and mitochondrial phosphorylation are abolished, demonstrating at least two kinase targets that are each sufficient to induce its dissociation from the complex. This likely explains why the extensive phosphorylation of Milton we observed was nonetheless not essential for the dissociation of the motors. Furthermore, Aurora A was sufficient to induce kinesin release but when mitotic cells were treated with an Aurora inhibitor, kinesin was still shed. This argued that another kinase, though not CDK1 or PLK1, was also sufficient to release kinesin. This interplay of CDK1 and Aurora A on mitotic mitochondria was also seen in fission fusion dynamics (Kashatus et al., 2011).

The redundant mechanisms for motor shedding may reflect its importance to the cell. We found that prevention of motor release induced asymmetric mitochondrial inheritance, mitotic delay, and formation of binucleate cells. The degree of asymmetric mitochondrial distribution at metaphase depended on when dynein was restored to the mitochondrial surface by rapalog addition. A likely explanation for the time dependence is the timing of centrosome migration (Figure 2.60). If mitochondria collapse around the microtubule-organizing center prior to centrosome migration, most mitochondria will remain associated with the extensive microtubule
array of the stationary centrosome and fewer will follow the migrating centrosome to the opposite pole, thereby producing an asymmetry. Thus the asymmetry was pronounced when rapalog was added during G2 or prometaphase but minor when added during metaphase, when the two centrosomes have separated and microtubules are again symmetric in the cell. The requirement for motor shedding to accomplish symmetric mitochondrial inheritance suggests that shedding may be regulated when asymmetric inheritance is required. A bias, for example, has been reported recently in inheritance of young versus old mitochondria in dividing stem cells (Katajisto et al., 2015). Differences in the degree of dynein retention on mitochondria could determine their segregation during asymmetric or selective divisions.

**Figure 2.60**

Kinesin attachment also changed mitochondrial distribution during metaphase, pushing them towards the periphery. If mitochondria attached to spindle microtubules, the plus-end directed movement of kinesin could move them towards the center of the spindle, and a minority of mitochondria occasionally were found there. That the mitochondria instead were
overwhelmingly peripheral is likely due to the fact that they would be more likely to encounter an astral than a spindle microtubule. Kinesin attachment also created an asymmetric metaphase distribution of mitochondria, but this effect was independent of the time of rapalog addition. The asymmetry might be produced by stochastic attachment of mitochondria to astral microtubules whose plus ends in the cellular periphery do not have a consistent location as the microtubules extensively grow and shrink.

We found that the asymmetry of mitochondrial distribution during metaphase could result in asymmetric mitochondrial inheritance. Examples of asymmetry at telophase were observed whether dynein or kinesin had been reattached. However, the asymmetries were less severe than at metaphase, suggesting that compensatory mechanisms occur to correct mitochondrial inheritance during anaphase/telophase. Mitochondrial-targeted myosin XIX influences mitochondrial inheritance primarily during anaphase/telophase and may accomplish this compensation because loss of myosin XIX causes asymmetric inheritance (Rohn et al., 2014). Further, Cenp-F has been found bound to Miro late in mitosis and may be another compensatory mechanism to ensure symmetric mitochondrial inheritance (Kanfer et al., 2015). In addition our analysis may be biased by selecting for cells that were able to proceed into telophase. Dynein attachment caused a delay in mitosis (Figure 2.52) and failure of cytokinesis as measured by binucleate cells (Figure 2.55). Thus the asymmetries we observed late in mitosis are a likely underestimate of the consequences of failure to undergo motor shedding.

Mitotic delay was one of the most pronounced consequences of attaching dynein to mitochondria and thereby linking them to the mitotic spindle (Figure 2.55). This arrest of mitosis was not observed with kinesin recruitment to mitochondria and thus correlates with spindle localization rather than just microtubule association or asymmetric distribution. Failure to
complete mitosis explains also the prevalence of binucleate cells when dynein, but not kinesin, was recruited to the mitochondria. We considered two hypotheses to explain the mitotic failure: 1) steric interference of the bulky mitochondria with correct spindle function or 2) depletion of endogenous free dynein required for other mitotic functions. Knockdown of dynein/dynactin components or sequestration of endogenous dynein by the viral E7 protein can delay mitosis (Nguyen and Munger, 2009; Raaijmakers et al., 2013). However, the delay we observe is more likely to result from mitochondrial attachment to the spindle because it was not seen in the absence of rapalog, although the same level of BICD was present to bind dynein. In addition, equivalent BICD expression and recruitment to peroxisomes, a smaller organelle, did not produce equivalent failure of mitosis (Figure 2.54-2.55). The results with peroxisome recruitment are consistent with previous studies in which smaller organelles like early endosomes and fragmented Golgi vesicles attach to microtubules without defects to cytokinesis (Dunster et al., 2002; Jongsma et al., 2015). In contrast, ER recruitment to the spindle through a mechanism that should not have altered dynein availability, did produce binucleate cells (Figure 2.54-2.55). Thus the failure to exit mitosis and the presence of binucleate cells correlates well with the presence of large organelles on the spindle that can interfere with chromosome segregation.

These studies have identified a phosphorylation-driven mechanism that causes motor proteins to be shed from the mitochondrial surface (Figure 2.61). By selectively severing the association of mitochondria with microtubules at the time of spindle formation, mitochondria are permitted to take up a peripheral location during mitosis. This redistribution has essential consequences for the completion of the cell cycle. Motor shedding facilitates symmetrical distribution and inheritance of mitochondria while also clearing the spindle of an organelle that
could arrest mitosis and prevent cytokinesis. Because phosphorylation releases the motors from the organelle but do not inhibit their microtubule interactions, kinesin and dynein remain free to serve their proper mitotic functions.

Figure 2.61

Figure 2.61 Graphical diagram of findings in Chapter 2
Experimental Procedures

Plasmid Constructs
For the sources of published plasmids and PCR-based construction of additional plasmids, see Extended Experimental Procedures.

Cell Culture and Transfection
HeLa, COS7, and HEK293T cells were cultured in DMEM containing L-glutamine, penicillin and streptomycin (Life Technologies), and 10% FBS (Atlanta Premium). Rat embryonic fibroblasts were cultured in the same media, but with 20% FBS. Plasmid transfections were performed with Lipofectamine2000 (Life Technologies) 2 days prior to experiments.

Cell Synchronization and Drug Treatments
Cells were treated with medium containing 2 mM thymidine for 16 h and then released into fresh media for 8 hours followed by a second 16 h thymidine incubation followed by fresh media for 8 hours for mitosis imaging, 10 hours for telophase imaging, or 16 hours for G1 imaging. For biochemistry, cells were synchronized by treating with either 100 ng/mL nocodazole or 5 µM s-trityl-L-cysteine (STLC) for 16 hours instead of the second thymidine block. For actin experiments, cells were treated for 10 minutes prior to fixation with 5 µm Latrunculin A or the vehicle control DMSO. For kinase inhibition, cells were treated with 10 µM RO-3306 or 2 mM ZM447439 for 16 hours.
Immunofluorescence and Protein Analysis

Cells were fixed using the following protocol optimized for microtubule stability: wash with PBS (phosphate buffered saline) and treat for 10 min with the buffer of 3% paraformaldehyde, 0.1 M PIPES, 1 mM MgCl2, 100 mM EGTA, 0.05% saponin. Wash three times with PBS and block with a buffer containing 3% bovine serum albumin (BSA), 0.1 M PIPES, 1 mM MgCl2, 100 mM EGTA, 0.05% saponin. Cells were stained with antibodies (see Extended Experimental Procedures) and imaged by confocal microscopy. Images were processed using ImageJ with linear adjustments to color and contrast. HeLa lysates were prepared similar to a previously used protocol (Glater et al., 2006). For immunoprecipitation and calf-intestinal phosphatase experiments see Extended Experimental Methods.

Statistical Analysis and Image Quantification

Statistical analysis was performed using GraphPad Prism v6.0e for MacOSX. Normality was determined using the D’Agostino & Pearson omnibus normality test. Solid bar graphs (percent overlap, percent binucleate cells) as well as percent mitotic cells are expressed as the mean ± SEM. Student’s t-test was used to determine the p value between the control and experimental conditions. For overlap calculations, the integrated density of immunofluorescence signals of mitochondria and tubulin were used to calculate the percent overlap. For mitochondrial overlap, the tubulin signal was thresholded by mean to create a mask to measure the overlapping mitochondrial signal, which was then divided by the total mitochondrial signal to create a percentage. For percent mitotic cells, 10 fields at 40x were captured at each time point, and the percent of mitotic cells was calculated and averaged. For the percent of binucleate cells, the average percentage of binucleate cells was calculated for 3 sets of 30 transfected cells. Scatter
plots of asymmetric index for either metaphase or telophase are expressed as the median with bars extending to the first and third quartile. The Kolmogorov-Smirnov test was used to determine significance between the control and experimental conditions. p < 0.05 was considered significant.

**Plasmid Constructs**

The following DNA constructs were previously published and used in this study: Mito-DsRed and GFP-Tubulin (Clontech, Mountain View, CA); RFP-Sec61β (Shibata et al., 2008); eYFP-STIM1 wild type and eYFP-STIM1 10A (Smyth et al., 2012); DIC2-C-RFP (Blasier et al., 2014); pEGFPC2-p150 (Moughamian and Holzbaur, 2012); HA-Aurora A K162R and HA-Aurora A T288D (Kashatus et al., 2011); Tom20-mCherry-FKBP (Miyamoto et al., 2012); HA-KIF5B MD-FRB, HA-BICD2-FRB, PEX-RFP-FKBP (Kapitein et al., 2010); GFP-Mito and GFP-BICD2-Mito (Hoogenraad et al., 2003); KHC-eCFP (Cai et al., 2007); myc-hMilton1 and HA-Miro1 (Pekkurnaz et al., 2014). To create the KIF5C motor domain chimera, the C-terminal domain of hMiro1 (560-end) was PCR amplified into XbaI/SpeI restriction sites inserted into the KIF5C-tdTomato construct, a gift from G. Banker (Jacobson et al., 2006) using the same restriction sites. Milton 28A was synthesized (GenScript, Piscataway, NJ) with the 28 alanine mutations (Figure S4A) in a pCMV tag 3A vector in order to match with the wild type construct used in our studies (Pekkurnaz et al., 2014).

**Immunoreagents**

For immunofluorescence of HeLa, COS7, and HEK293T cells, and rat embryonic fibroblasts, the following antibodies were used: anti-Tom20 at 1:500 (FL-145, Santa Cruz Biotechnology, Inc.),
anti-tubulin at 1:1000 (clone DM1A, Sigma-Aldrich), anti-GFP 1:500 (Aves Labs, Inc.), anti-myc at 1:500 (9E10, Santa Cruz Biotechnology, Inc.), anti-HA 1:500 (Cell Signaling). For secondary antibodies, the following were used: Alexa-488, Cy5, or Cy3 conjugated anti-mouse and anti-rabbit antibodies at 1:500 (Molecular Probes, Invitrogen). DNA was visualized by Hoechst 33342 (Vybrant Apoptosis Assay Kit #5, Molecular Probes, Invitrogen) using the manufacturer’s protocol.

In addition to the aforementioned antibodies, the following were used exclusively on immunoblots: anti-DIC 1:1000 (Millipore), anti-KHC 1:100 (clone H2, Millipore), anti-p150 1:1000 (BD Biosciences), anti-hMilton (TRAK1) at 1:1000 (Sigma-Aldrich), anti-Rhot1 (Miro) at 1:1000 (clone ARP44817, Aviva Systems Biology), anti-Cyclin B 1:1000 (Santa Cruz Biotechnology, Inc.), anti-ATP5β 1:5000 (Sigma-Aldrich), anti-CDK1 substrates 1:1000 (Cell Signaling). HRP conjugated anti-rabbit and anti-mouse secondary antibodies (Jackson, ImmunoResearch Laboratories, Inc.) were used for chemiluminescent detection using SuperSignal West Dura (Pierce Biotechnology, Thermo Scientific) by either using film or the ImageQuant LAS 4000 mini.

**Live-Cell Imaging**

HeLa cells were transfected with Mito-dsRed and GFP-tubulin prior to synchronization. Cells were incubated with HEPES containing DMEM without phenyl red. Z-stacks were spaced at 0.55 µm per slice. Videos were prepared using the composite z series without the first 1 µm in order to visualize the spindle better.
Biochemical Assays

For immunoprecipitation (Figure 3C), HEK293T cells were transfected with myc-Milton and synchronized using thymidine and STLC. myc-hMilton1 was immunoprecipitated as previously (Glater et al., 2006) with anti-myc (Novus).

For mitochondrial isolation, 3 million cells were suspended in an isolation buffer containing 200mM sucrose, 10mM Tris pH 7.4, 1mM EGTA and subsequently homogenized with a Dounce homogenizer. Lysates were centrifuged at 2000 g for 10 minutes to remove cellular debris and the supernatant was centrifuged at 10,000 g for 20 minutes to pellet mitochondria. For incubations with cytosol, the remaining cytosolic supernatant from interphase or mitotic cells was subject to an additional centrifugation at 16,000 g for 20 minutes to insure that no mitochondria were carried over. Mitochondrial fractions were incubated with the appropriate cytosol for two hours on the rotator at 4°C. Mitochondria were reisolated at 10,000 g for 20 minutes. For treatment with calf intestinal phosphatase (CIP), 10x Buffer 2 (New England Biosciences) was added to mitochondrial isolation buffer along with Protease Inhibitor Set III (Calbiochem), 100 µM PMSF, and 35 units of CIP (New England Biosciences) per 50 µL. For the inactive CIP condition, 20 mM Na$_3$VO$_4$ was preincubated with CIP for 10 min. After 1 hour CIP incubation at 37 degrees Celsius, Na$_3$VO$_4$ was added for 10 min to inactivate the phosphatase. Incubation with the appropriately treated cytosol occurred for 2 hours at 4°C and mitochondria were reisolated at 10,000 g for 20 minutes. Mitochondria immunoprobed for the indicated proteins.

For CDK1 assays, isolated mitochondria were resuspended in mitochondrial isolation buffer with 10x Protein Kinase Buffer (New England Biosciences), 5 mM Na$_3$VO$_4$, 100 µM PMSF, Protease Inhibitor Set III, and 1 mM ATP with or without 1000 Units of CDK1 (New
England Biosciences) per 100 µL. Mitochondria were incubated at 30°C for 2 hours and after addition of 500 µL of fresh mitochondrial isolation buffer were reisolated at 10,000 g for 20 minutes to separate from the disassociated motors. Mitochondria were immunoprobed for the indicated proteins.

For rapalog treatments, HeLa cells expressing the appropriate constructs were treated with 5 µM of the A/C Heterodimizer (Clontech) or ethanol, the vehicle control at the indicated times.

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Chapter 3: Mitochondrial Positioning during G2 Determines Positioning during Mitosis
Introduction

Following the work done in Chapter 2, we sought to elucidate unanswered questions that the study left open. We investigated the effects of exogenous and endogenous motor recruitment as well as the effects of G2 mitochondrial positioning on inheritance and distribution. This chapter is not delineated based on data, interpretation, and methods; rather, each subsection has the required information to understand the conclusion in its entirety. Also to prevent redundancy, only methods not used in the previous chapter are included in this chapter.

Kinesin drives peripheral mitochondrial movement via astral microtubules

During mitosis, mitochondria are devoid of motors, but through use of FRB/FKBP constructs, we are able to force dynein and kinesin motors back onto the mitochondrial surface. When dynein was recruited, mitochondria reattached onto the mitotic spindle and preferentially localized toward the poles. This was expected since the minus ends of microtubules are located at the poles. For the plus ends of microtubules, there are two locations during mitosis. They are near the metaphase plate (kinetochore and interpolar microtubules) and at the cell periphery (astral microtubules).

In our experiments that exogenously attached kinesin to mitochondria, we were surprised that only three out of ninety analyzed cells had mitochondria localized to the metaphase plate whereas the majority of cells had mitochondria pushed more peripherally. We tested whether this movement was due to astral microtubule attachment. Since these microtubules are quite dynamic, it was difficult to see mitochondria attached to astral microtubules during live cell imaging, so we implored a selective destabilization using a cold treatment or a low level of nocodazole. Both treatments only destabilized astral microtubules while keeping the mitotic
spindle intact. We had more success using low levels of nocodazole to depolymerize astral microtubules and moved forward with those experiments.

**Figure 3.1**

HeLa cells transiently expressing Tom20-mCherry-FKBP and HA-KIF5B MD-FRB were synchronized and treated with DMSO or nocodazole to depolymerize astral microtubules prior to treatment with either ethanol (control) or rapalog for 10 minute. Rapalog caused mitochondria to assume a more peripheral localization, but if astral microtubules were depolymerized prior, mitochondria did not move peripherally. **Figure 3.2** Averaged radial distribution and percent overlap of mitochondrial and tubulin signals for 30 rapalog treated cells with or without astral microtubules. Scale bars represent 5 microns.

We tested three conditions: DMSO + ethanol, DMSO + rapalog, and nocodazole + rapalog. Ethanol treatment was the vehicle control and would create normal mitochondrial localization whereas rapalog treatment would push mitochondria to the periphery. DMSO was the vehicle control for the nocodazole treatment. HeLa cells were transfected with HA-KIF5B MD-FRB and TOM20-mCherry-FKBP constructs as before. As expected, the DMSO + ethanol had a regular distribution of mitochondria, and the DMSO + rapalog condition pushed the mitochondria towards the periphery. Prior to rapalog/ethanol treatment, cells were treated with a low level of nocodazole (50 nM for 30 min) or DMSO. In the nocodazole treatment, there was a
decrease of astral microtubules. As expected, the mitochondria were less peripheral in the nocodazole + rapalog treatment compared to the DMSO + rapalog treatment, but they were still somewhat peripheral compared to the DMSO + ethanol control (Figure 3.1-3.2). This is likely due to the inability to fully eliminate all the astral microtubules.

**Figure 3.3**

![Figure 3.3 Image](image)

**Figure 3.4**

Composite | Tubulin | Mitochondria | KIF5B/BICD
---|---|---|---
Control | | | |
Rapalog | | | |

**Figure 3.3** HeLa cells transiently expressing Tom20-mCherry-FKB (magenta), HA-KIF5B MD-FRB, and HA-BICD-FRB (gray) treated with rapalog for 10 minute. Majority of mitochondria localized perinuclearly with some mitochondria in the periphery (see arrows). DNA (blue), mitochondria (magenta), tubulin (green), motors (grey). **Figure 3.4** Cells transfected as in Figure 3.3 were synchronized and treated with either ethanol (control) or rapalog. Majority of mitochondria attached to the spindle when treated with rapalog. Scale bars represent 5 microns.
Exogenous dynein and kinesin attachment to mitochondria induces spindle attachment

We were curious to know what would happen if both kinesin and dynein were attached to mitochondria during mitosis. HeLa cells were transfected with TOM20-mCherry-FKBP FRB-motors except the amount was equally divided between HA-BICD2-FRB and HA-KIF5B MD-FRB constructs. Upon rapalog addition during interphase, mitochondria were clustered into two distinct populations with the majority located perinuclearly and the minority in the periphery (Figure 3.3). When rapalog was added during mitosis, mitochondria were more exclusively on the spindle (Figure 3.4). Out of ninety cells analyzed, only five contained mitochondria in the periphery. We concluded that dynein recruitment was likely stronger than kinesin attachment. However, this could have multiple explanations including differences in recruitment kinetics, strength of motors, and localization of motors prior to rapalog treatment.

Dynein recruitment affects spindle angle

During mitosis, dynein motors provide essential functions during cell division such as chromosomal attachment and movement, spindle angle and organization, and aiding in the spindle assembly checkpoint. In our rapalog system, we recruited endogenous dynein to mitochondria by exogenous BICD attached to the mitochondrial surface. During our analysis, we noticed that there were other phenotypic changes among them including binucleate cell formation, mitotic delay, and asymmetric inheritance of mitochondria. These phenotypes were found to be due to mitochondrial attachment to mitotic spindle and not due to recruitment of dynein since overexpression of HA-BICD-FRB during the vehicle treatment was unable to invoke the same changes. Further, when we recruited comparable levels of dynein to peroxisomes, binucleate cell formation and mitotic delay did not occur. However, we did find a
phenotype that was consistent in both rapalog-induced recruitment of dynein to either mitochondria or peroxisomes; spindle angle of the poles changed relative to the coverslip. Driven by dynein, the poles normally divide parallel to the coverslip (Bergstralh and St Johnston, 2014). In metaphase, HeLa cells transiently expressing Tom20-mCherry-FKBP and HA-BICD-FRB, the spindle angle changed from the average 5.47 degrees during ethanol treatment (vehicle control) to 17.65 in rapalog-treated cells (Figure 3.5). This effect was consistent when dynein was recruited to peroxisome through rapalog treatment (data not shown). Additionally, there was no change when kinesin was attached to mitochondria due to rapalog treatment (Figure 3.6). Overall, spindle angle changed due to recruitment of endogenous dynein preventing it from doing its normal function. This same phenotype is seen in other studies. Knockdown of certain dynein and dynactin components change spindle angle as well (Nguyen and Munger, 2009; Raaijmakers et al., 2013).

**Figure 3.5** HeLa cells transiently expressing Tom20-mCherry-FKBP and HA-BICD-FRB were synchronized and treated with either ethanol (control) or rapalog for 10 minute. Rapalog caused the spindle angle to significantly change compared to the vehicle control. **Figure 3.6** HeLa cells transiently expressing Tom20-mCherry-FKBP and HA-KIF5B MD-FRB were synchronized and treated with either ethanol (control) or rapalog for 10 minute. There was no significant change in spindle angle between the two treatment conditions.
Mitochondrial positioning during metaphase is dependent on distribution during G2

Passive inheritance requires that the organelles occur in large enough quantity, even and random distribution throughout the cytoplasm, and an equal inheritance of cytoplasm volume into each of the daughter cells. Through our study, we showed that mitochondria meet the criteria and are stochastically positioned during metaphase. Since mitochondria are mostly evenly distributed throughout the cytoplasm during G2, they remain evenly distributed throughout the cytoplasm as they release from their normal attachments (ER, actin, microtubules). We then asked ourselves whether or not affecting the distribution of mitochondria during G2 would affect distribution during metaphase and consequently affect inheritance. To test our hypothesis, we measured two parameters that could affect metaphase distribution: asymmetry and localization (peripheral/central). We made changes to mitochondrial distribution during G2 and saw how they affected metaphase distribution.

There was evidence of this phenomenon in our previous work and in the work of others. From experiments performed by Pekka Katajisto, he found that mitochondria segregate asymmetrically during stem cell/progenitor cell division (Katajisto et al., 2015). Mitochondria with older proteins tended to be inherited by cells that would become more differentiated; cells that maintained stem-like nature inherited mitochondria with younger proteins. During G2, younger mitochondria were localized in the periphery, and older mitochondria were localized near the nucleus. Disruption to this localization also disrupted asymmetric inheritance. Using a DRP-1 inhibitor MDIV-1, the mitochondrial network became fused, and distinct subdomains of young versus old mitochondria were no longer maintained. As a result, the asymmetric division was also disrupted. It is not known whether this disruption is due to affecting the mitochondrial network or interfering with the segregation of old/young proteins.
Figure 3.7 HeLa cells transiently expressing Tom20-mCherry-FKBP and HA-BICD2-FRB were treated with ethanol (control) or rapalog at G2, prometaphase, or metaphase and imaged by confocal microscopy at metaphase. The asymmetric index of metaphase cells was calculated on 3D projections of treated cells for BICD-FRB transfected cells. Figure modified from Figure 2.46. Figure 3.8 Diagram of asymmetry from rapalog treatment.

As seen in the previous chapter, there was a difference in mitochondrial asymmetry severity dependent on time of rapalog addition. In cells transfected with the HA-BICD2-FRB and TOM20-mCherry-FKBP, mitochondria had a high amount of asymmetry during metaphase when rapalog was added early during G2 compared to later rapalog addition during metaphase (Figure 3.7). When imaged during G2, the cells treated with rapalog had mitochondria localized specifically to the microtubule-organizing center, which created an asymmetric, perinuclear bias (Figure 3.8). Hence, the even distribution of mitochondria during interphase was disrupted, and this resulted in downstream effects during metaphase. Further, we tested this same phenomenon
by attaching motors to peroxisomes, which have the advantage of being without motors
normally.

In HeLa cells, peroxisomes are spread throughout the cytoplasm during interphase
(Figure 3.9). During metaphase, peroxisomes likewise are spread throughout the cytoplasm
though less symmetrically than mitochondria, likely due to their decreased number. We localized
peroxisomes to the microtubule-organizing center by transfecting HeLa cells with Tom20-
mCherry-FKBP and HA-BICD2-FRB and treated with rapalog. Similar to mitochondria
conditions, peroxisomes were asymmetrically and perinuclearly biased during interphase. When
these cells proceeded into mitosis, asymmetry persisted. Control cells had an average asymmetry
index of 0.42 compared to rapalog-treated cells that had an average index of 0.52 (Figure 3.10-
3.11).

As another form of disruption, we transfected HeLa cells with kinesin heavy chain and
Milton; it had long been known in the lab that overexpression of kinesin heavy chain and Milton
would mislocalize mitochondria to the periphery in HeLa cells. We used this perturbation to see
if mitochondria would remain peripheral during metaphase. We verified that overexpressed
kinesin detached from mitochondria during mitosis through an immunoprecipitation. During G2,
the mitochondria were pushed to the periphery as expected (Figure 3.12). When observed during
mitosis, mitochondria still remained peripheral (Figure 3.13). We concluded that perturbations
during G2 could persist into mitosis and affect inheritance.
Figure 3.9 (modified from Figure 2.54). HeLa cells were transfected with PEX-RFP-FKBP and HA-BICD-FRB (Peroxisome BICD) and subsequently treated with ethanol (control) or rapalog. Cells were imaged during interphase. Figure 3.10 HeLa cells were transfected as in Figure 3.9 and were treated with ethanol (control) or rapalog before cells entered mitosis. Cells were imaged during metaphase. Figure 3.11 Asymmetric indices of mitochondria in 30 metaphase cells with a p value of 0.03. Data are represented as median with the interquartile range. Scale bars represent 5 microns.
Figure 3.12 HeLa cell were transfected with Milton and were also transfected with KIF5B in experimental conditions. During interphase, Milton and KIF5B expression pushes mitochondria to the periphery. Figure 3.13 Mitochondria remain peripheral during metaphase in experimental conditions. Scale bars represent 5 microns.
Figure 3.14 HeLa cells were treated with 100 µM of CCCP or DMSO (vehicle control). Mitochondria (magenta) distribution was observed in relationship to microtubules (green) and DNA (blue). CCCP disrupted distribution and symmetry. Figure 3.15 Cells treated as in Figure 3.15 were imaged during metaphase. Figure 3.16 Asymmetric index was calculated for cells treated with DMSO (control), 10 µM, and 100 µM of CCCP. Cells treated with 10 µM CCCP had a lesser change in mitochondrial distribution and likewise had a lesser effect during metaphase. Scale bars represent 5 microns.

When mitochondria are damaged, Miro is degraded in a Pink1/parkin dependent fashion. Motors are released from the mitochondrial surface, and mitochondria naturally migrate towards the microtubule-organizing center when damaged (Wang et al., 2011). To bias mitochondria to the perinuclear region, cells were treated with the depolarizing agent CCCP or the mitochondrial respiratory inhibitor antimycin A. CCCP proved to have a more dramatic effect on positioning of
Figure 3.17 HeLa cells were transfected with syntaphillin wildtype or syntaphillin lacking the microtubule binding domain (ΔMTB). Mitochondria were forced perinuclear. Figure 3.18 Cells transfected as in Figure 3.17 were followed into mitosis, but distribution was significantly changed compared to syntaphillin ΔMTB. Scale bars represent 5 microns.

mitochondria during G2. The mitochondrial network collapses, and mitochondria are localized to the mitotic organizing center upon CCCP treatment for 4 hours when compared to DMSO controls (Figure 3.14). During mitosis, the mitochondria did not appear to be localized onto the spindle, but there was a high amount of asymmetry during the CCCP treatment (Figure 3.15-
The persistent asymmetry from G2 was similar to the rapalog/dynein experiments, but unlike the rapalog experiments, the mitochondria not could attach to the spindle nor maintain their central distribution. Presumably this is due to mitochondria being able to attach to the spindle because of exogenous dynein attachment, but in the CCCP experiments, mitochondria are lacking all motor attachment and cannot attach to microtubules. This conclusion is further demonstrated by expression of syntaphillin in HeLa cells. Syntaphillin is expressed in the axonal compartments of neurons and stops transport of mitochondria (Kang et al., 2008). When expressed in HeLa cells, mitochondria localize perinuclearly but not exclusively at the microtubule-organizing center, unlike CCCP treatment or rapalog/dynein-induced localization. Upon entering mitosis, the syntaphillin-expressing cells have normally distributed mitochondria. Similar to the CCCP experiments, mitochondria were perinuclear prior to mitosis but because of motor shedding, mitochondria were unable to stay near the spindle apparatus.

From these experiments, we concluded that effects to G2 mitochondrial distribution can impact symmetry and localization of mitochondria during mitosis. The kinesin overexpression data show that induced peripheral distribution of mitochondria remains during mitosis. This not only supports our claim that mitochondrial position during mitosis is a passive effect (were it an active mechanism, the mitochondria would have been corrected for their displacement), but it also shows the importance of mitochondrial distribution prior to mitosis. Using peroxisomes, we were able to create asymmetry during mitosis by first inducing asymmetric, perinuclear clustering during G2 similar to the mitochondrial phenotype. Furthermore, asymmetry induced during G2 also persists during mitosis as seen with the CCCP experiments. CCCP treatment causes mitochondrial clustering at the microtubule-organizing center similar to rapalog-induced attachment of dynein on the mitochondrial surface. In both cases, asymmetric mitochondrial
distribution occurs during mitosis. However, CCCP was unable to mislocalize mitochondria onto the spindle, likely due to a lack of motors that would attach mitochondria and microtubules. Additionally, there are forces that naturally push objects away from the mitotic spindle in order to allow for the congression of chromosomes towards the metaphase plate (Levesque and Compton, 2001). These “polar forces” may be responsible for pushing mitochondria away from the spindle despite their G2 localization.

**Cell Culture and Transfection**

HeLa cells were cultured in DMEM containing L-glutamine, penicillin and streptomycin (Life Technologies), and 10% FBS (Atlanta Premium). Plasmid transfections were performed with Lipofectamine2000 (Life Technologies) 2 days prior to experiments.

**Cell Synchronization and Drug Treatments**

Cells were treated with medium containing 2 mM thymidine for 16 hours and then released into fresh media for 8 hours followed by a second 16 hours thymidine incubation followed by fresh media for 8 hours for mitosis imaging. For astral microtubule depolymerization, cells were treated with 50 nM nocodazole for 30 min prior to rapalog treatment.

**Plasmid Constructs**

The following DNA constructs were previously published and used in this study: Mito-DsRed and GFP-Tubulin (Clontech, Mountain View, CA); RFP-Sec61β (Shibata et al., 2008); eYFP-STIM1 wild type and eYFP-STIM1 10A (Smyth et al., 2012); DIC2C-RFP (Blasier et al., 2014); pEGFPC2-p150 (Moughamian and Holzbaur, 2012); HA-Aurora A K162R and HA-Aurora A
T288D (Kashatus et al., 2011); Tom20-mCherry-FKBP (Miyamoto et al., 2012); HA-KIF5B
MD-FRB, HA-BICD2-FRB, PEX-RFP-FKBP (Kapitein et al., 2010); GFP-Mito and GFP-
BICD2-Mito (Hoogenraad et al., 2003); KHC-eCFP (Cai et al., 2007); myc-hMilton1 and HA-
Miro1 (Pekkurnaz et al., 2014). To create the KIF5C motor domain chimera, the C-terminal
domain of hMiro1 (560-end) was PCR amplified into XbaI/SpeI restriction sites inserted into the
KIF5C-tdTomato construct, a gift from G. Banker (Jacobson et al., 2006) using the same
restriction sites. Milton 28A was synthesized (GenScript, Piscataway, NJ) with the 28 alanine
mutations (Figure S4A) in a pCMV tag 3A vector in order to match with the wild type construct
used in our studies (Pekkurnaz et al., 2014).
Chapter 4: Discussion
**Introduction**

Throughout my dissertation, I set out to understand the mechanisms behind why and how mitochondria detach from microtubules during cell division. Disruption to mitochondrial detachment revealed insights into how mitochondria are inherited by dividing cells.

**Mitochondrial release from microtubules during cell division**

There have been alternative hypotheses to why mitochondria do not attach to microtubules during mitosis: they are attached to other tethers like actin, ER, or intermediate filaments that pull mitochondria off of the spindle (Lee et al., 2007); mitochondria are pushed away from the spindle due to “polar wind” forces and the density of the spindle prevents attachment; changes to microtubules prevent mitochondrial attachment. We found that mitochondria are released from microtubules through release of dynein and kinesin motor proteins from the mitochondrial surface. Lee et al had also observed dynein intermediate chain and p150 release from mitochondria, but it was unclear whether this dissociation was responsible for mitochondrial release from microtubules (Lee et al., 2007). Likewise, we confirmed DIC and p150 released from mitochondria, and we found that kinesin released during mitosis as well. We went on to show that exogenous attachment of motors was sufficient to change mitochondrial distribution as well as attachment onto microtubules. Given our data, we showed that motor dissociation is necessary for detachment from the spindle. Through these experiments, we also ruled out alternative hypotheses. Since mitochondria can attach to microtubules through motors, we concluded that there are no inherent microtubule changes that prevent attachment. The “polar wind” hypothesis may still be valid although it is clear that motor attachment is strong enough to overcome the “polar wind” forces.
We found that motors release due to phosphorylation by cyclin B-CDK1 for dynein and Aurora A for kinesin. Phosphorylation of mitochondrial and cytoplasmic proteins were responsible for dynein release during mitosis; whereas, kinesin release was dependent on proteins in the cytoplasmic fractions. Dynein release from mitochondria appears to be consistent among other membranous structures during cell division. In Xenopus Golgi membrane preparations, dynein and dynactin components were seen to dissociate from membranes during mitosis compared to interphase. In motility assays, it was seen that Golgi membranes from mitotic fractions had decreased transport along microtubules compared to interphase fractions, but this motility was restored by incubation with interphase cytoplasm (Allan and Vale, 1991; Dell et al., 2000; Niclas et al., 1996). Likewise incubation of membranes with cyclin B-CDK1 resulted in decrease of dynein attachment in Xenopus extracts and serine 197 of dynein light intermediate chain was identified as a potential regulator for dynein control (Addinall et al., 2001; Dell et al., 2000). We tested phosphoresistant mutants for S197 on dynein light intermediate chain, and we also tested phosphoresistant mutants for dynein intermediate chain that were previously shown to regulate mitochondrial motility (Blasier et al., 2014). Neither of these proved to be sufficient to prevent dynein dissociation during mitosis (data not shown). This sheds more importance on our studies, which show there are multiple phosphorylation targets for dynein release. In our phosphatase experiments, dynein reattached to the mitochondrial surface only when both the mitochondrial and cytosolic fractions were treated with phosphatase, indicating that there are at least two targets of CDK1. Additionally, there are multiple proteins of the dynein-dynactin complex known to be phosphorylated during mitosis and at multiple amino acids (Olsen et al., 2010), thus, making the problem of determining the causative phosphorylations even more complex.
One interesting area of dissociation may be the connection between dynactin and dynein. In our experiments, it is true that p150 and DIC both dissociated from the mitochondrial surface, but p150 would only dissociate about 75% of the original level whereas DIC would dissociate 95%-100% compared to interphase. Thus it is possible that the dissociation of dynein may be at the interface between dynein and dynactin. In fact, dynein and dynactin proteins have different functions during mitosis as seen through a systematic knocked-down (Raaijmakers et al., 2013). For instance, dynactin was not required for dynein dependent spindle organization. If the regulation point was at dynein-dynactin interface, this may explain how they can localize and perform distinct functions during cell division.

Mitochondria are released from microtubules to prevent cytokinetic defects. From our study, we observed cytokinesis failure, delay of the spindle assembly checkpoint, and asymmetric inheritance of mitochondria. Motors were found to be the important tethers between mitochondria and microtubules, but motors likely release from mitochondria in order to fulfill other roles during mitosis. When endogenous dynein was recruited to mitochondria, we observed various phenotypes that could be a result of dynein mislocalization. In particular, the mitotic spindle angle changed when compared to controls, and the time spent in metaphase was lengthened as well. Both of these phenotypes were seen by knocking down dynein subunits or by sequestering dynein improperly to human papillomavirus E7 protein (Nguyen and Munger, 2009; Raaijmakers et al., 2013). As a proof of principle, we recruited dynein to peroxisomes, which also affected spindle angle but had a lesser effect on timing in metaphase. We found that steric hindrance by the larger mitochondrial network on the spindle was responsible for cytokinesis failure and mitotic delay.
Unfortunately, our rapalog system did not recruit endogenous kinesin heavy chain to mitochondria. Instead, we over-expressed a FRB-kinesin motor domain. It is not clear if kinesin has a role in mitosis and if endogenous recruitment of kinesin to mitochondria during cell division would result in other cytokinetic defects. In female mouse meiotic cell division, knock down of KIF5B through RNAi caused a delay in germinal vesicle breakdown and defects in the first polar body extrusion (Kidane et al., 2013). In mitotic cells, the KIF5B knockdown caused an overamplification of centrosome and defects in chromosomal segregation. In null KIF5B -/- mice, homozygotes were nonviable and had lethality between 9.5 and 11.5 days postcoitum (Tanaka et al., 1998). The cause of the defects was not clear. Mice embryos were at least able to go through several rounds of cell division. Although it is unclear whether kinesin heavy chain has other roles during mitosis, our data show that kinesin attached to mitochondria induces an increased amount of asymmetry during cell division.

Mitochondria are transported to the cleavage furrow through a kinesin/Miro dependent process (Lawrence et al., 2016). This fits nicely with our data. A possible model is that mitochondria have motors attached to them before mitosis, but these motors release due to mitotic kinases. Upon anaphase onset, phosphatases dephosphorylate motors and allow them to reattach. Dephsophorylated kinesin heavy chain would reattach to mitochondria and move them towards the cleavage furrow. This hypothesis is supported by the CENP-F directed movement during cytokinesis. Mitochondria were found to associate with the growing ends of microtubules during G2 in addition to cytokinesis. This is through an interaction between Miro on the mitochondrial surface with CENP-F, which binds the plus-end directed microtubule associated protein EB1(Kanfer et al., 2015). Although peak association occurred during G2 and cytokinesis, there was a dramatic decrease of CENP-F mitochondrial localization during mitosis, supporting
our claim of the importance of mitochondrial-microtubule dissociation. Likewise, it follows a pattern that attachment precedes mitosis and reengages during anaphase and into cytokinesis, but during metaphase, mitochondrial attachments are phosphorylated preventing association with microtubules.

**Mitochondrial release from all attachments during mitosis**

Microtubule attachment was not the only attachment disrupted during mitosis. Actin and ER associations dissolve as demonstrated, showing that mitochondria distribution is unaffected when actin and ER localization is disrupted. Furthermore, when the spindle apparatus is disrupted through nocodazole treatment, mitochondria are able to fill in the space previously excluded by the spindle. This suggests that mitochondria are not tethered to other elements, rather, they are free-floating within the cytoplasm. Mitochondria can attach to actin filaments through myosin V and myosin XIX proteins (Pathak et al., 2010; Quintero et al., 2009). Myosin XIX was shown to play an important role in symmetric division of mitochondria during cytokinesis but did not appear to have a role during metaphase (Rohn et al., 2014). It must have some form of regulation to prevent mitochondria from attaching to the actin network, and phosphorylation is an attractive mechanism since it is easily induced during prometaphase and reversed during anaphase.

For ER attachment, mitochondria are known to associate heterotypically through MFN2 (de Brito and Scorrano, 2008). How these interactions are disrupted is not fully known, but they may involve similar types of regulations as microtubule detachment. During a preliminary screen of candidates, MFN2 western blots revealed that MFN2 was not degraded during mitosis (data not shown). Additionally, proteins of interest were not significantly different between interphase
and mitosis as measured by quantitative mass spectroscopy (Olsen et al., 2010). Phosphorylation is also a particularly attractive form of regulation since it can be reversed by a myriad of phosphatases during cytokinesis/G1.

**Interplay between mitochondrial transport and dynamics during mitosis**

Although a direct study to understand the relationship between mitochondrial fission/fusion dynamics and mitochondrial motility is lacking, studies have shown a potential indirect link between the two. In fact, some disease models like CMT2A and dominant optic atrophy have been known to exhibit similar phenotypes by affecting proteins involved in transport or fission/fusion (Misko et al., 2010). Altering fission/fusion dynamics through MFN2 or DRP-1 perturbations has shown to affect motility; likewise, affecting dynein was shown to increase mitochondrial fusion (Giedt et al., 2012; Varadi et al., 2004).

In our studies, we saw a change to mitochondrial dynamics by affecting the motor equilibrium. When we constitutively attached kinesin, mitochondria created abnormal circular, donut-shaped mitochondria. When dynein was constitutively attached to mitochondria, mitochondria became highly fused. In other studies, cytokinesis failure and mitotic delay were seen in cells in which fission was inhibited during mitosis. Cytokinesis failure was seen by disrupting either RALA or RALBP1, which were previously shown to be necessary to increase mitochondrial fission during mitosis through recruitment of DRP-1 (Kashatus et al., 2011). Likewise, inhibition of DRP1 induces a delay in cell cycle progression as well as aneuploidy through centrosomal overamplification (Qian et al., 2012). Misregulation of the fission-fusion dynamics also affects progress through the cell cycle. In particular, knocking down the mitochondrial fission protein hFis1 or the Mfn1 regulator MARCH5 induced abnormal
mitochondrial elongation and cellular senescence (Lee et al., 2014; Park and Cho, 2012). Furthermore, increasing mitochondrial fission saw the opposite effect. In Drosophila, inhibiting mitochondrial fusion through RNAi increased mitochondrial fragmentation and induced cell hyperproliferation as well as cyclin B levels (Gupte, 2015). MyoXIX knockout cells have an increased mitochondrial fusion as well as increased failure of cytokinesis as measured by multinucleate cell formation (Rohn et al., 2014). Interestingly, cytokinesis failure was abrogated by increasing mitochondrial division through MFN2 knockdown.

It is still unknown whether mitochondrial attachment to microtubules is sufficient to induce cytokinesis failure or if cytokinesis failure is a result of increased fusion. Likely steric hindrance of the scission machinery is the root cause of cytokinesis failure. We saw that ER attachment to the microtubule spindle also induced cytokinesis failure, but peroxisomes attachment did not have an effect. Hence, the amount of organelle on the spindle would drive cytokinesis failure; greater amounts of organelle would increase the amount of steric hindrance. Given the great amount of mitochondria, cytokinesis failure would potentially still occur, but an experiment knocking down fusion while still attaching mitochondria to the spindle is still required to fully support the hypothesis.

Using purified kinases, chemical inhibitors, and expression of constitutively active kinase constructs, we found that cyclin B-CDK1 releases dynein and Aurora A kinase releases kinesin from the mitochondrial surface. We were somewhat surprised to see that these kinases were also involved in the increased amounts of fission during mitosis (Cerveny et al., 2007; Kashatus and Counter, 2011; Kashatus et al., 2011; Taguchi et al., 2007; Yamano and Youle, 2011). RALA was found to be preferentially phosphorylated by Aurora A, which localizes RALA from the plasma membrane onto the mitochondria. Once on the mitochondrial membrane, RALA is able
to recruit RALBP1, which tethers CDK1 along with it. CDK1 then phosphorylates DRP-1 and increases its activity in a cell cycle dependent fashion. The recruitment of CDK1 by RALBP1 may also regulate the release of dynein from the mitochondrial surface. Knockdown of RALA/RALBP1 would likely give hints of whether this mechanism is at play in our results as well.

**Mitochondrial inheritance: active or passive?**

To date, most of the published work regarding mitochondrial inheritance has been focused primarily during cytokinesis; whereas our study focused on distribution and regulation during metaphase leading into cytokinesis (Kanfer et al., 2015; Lawrence et al., 2016; Lawrence and Mandato, 2013b; Rohn et al., 2014). According to these studies, inheritance seems to be driven actively. Taken together, mitochondrial inheritance appears to go through phases of active and passive regulation.

From our studies, we found that distribution during metaphase is a passive process ensured by release from microtubules, actin, and ER. To further show this, we saw that changes to mitochondrial distribution during G2 also affected symmetry and positioning during metaphase. If mitochondria were in fact positioned actively, then we would have expected mitochondrial symmetry during metaphase to be reestablished despite how the mitochondria were positioned prior to cell division. Instead, cells were unable to compensate for the dramatic positioning changes we induced. As the cell proceeded into cytokinesis, mitochondria were still asymmetrically inherited but to a lesser extent. If inheritance was completely passive, we would have expected the amount of asymmetry to persist, but if inheritance was also governed actively, the cell would be able to fully compensate and induce symmetric division. Given our data, we
concluded that the cell has mechanisms in place to reduce asymmetry although the initial
distribution of mitochondria has a dramatic role.

Studies looking at cytokinesis showed that mitochondria can be actively regulated
through CENP-F/Miro/EB1, Myosin XIX, and the motor adaptor complex. Mitochondria ride
along the growing tips of microtubules through attachment of Miro to CENP-F associated with
EB1 towards the midzone (Kanfer et al., 2015; Lawrence and Mandato, 2013b). This complex
likely regulates symmetric inheritance since disruption of the CENP-F/Miro interaction results in
some asymmetry. Kinesin-driven motility of mitochondria into the cleavage furrow may also
ensure symmetric inheritance by active means (Lawrence et al., 2016). Furthermore, myosin XIX
also plays a role during inheritance as disruption causes asymmetry (Rohn et al., 2014).
Mitochondrial inheritance, therefore, may initially be positioned and distributed passively during
the early stages of mitosis, but as the cell proceeds into cytokinesis, microtubule and actin based
attachments redistribute mitochondria to be inherited more evenly. While this is an attractive
model, it is important to note that the active phase of inheritance is not completely accurate.
Disruptions during metaphase by our system still resulted in asymmetric divisions. Furthermore,
it is not know how movement by mitochondria would ensure symmetric inheritance.

**Can mitochondrial inheritance be orchestrated?**

Mitochondrial inheritance in stem cells introduced the idea that mitochondrial inheritance
can be directed. Using snap-tag labeling, older and younger mitochondria were distinctly labeled
and tracked. These groups of mitochondria were not evenly inherited in stem cell populations.
Progenitor-like cells preferentially inherited older mitochondria whereas the stem-like daughter
cell inherited younger mitochondria (Katajisto et al., 2015). The mechanisms behind this
phenomenon are not known. Could directed inheritance occur from differences in motor attachment during mitosis? Could directed inheritance occur through changes in distribution during mitosis? Young, health mitochondria preferentially localize towards the periphery in stem cells whereas the older, damaged mitochondria group perinuclearly (Katajisto et al., 2015). As mitochondria age, they lose membrane potential, produce a higher amount of reactive oxygen species, and tend to group more perinuclear (Parihar and Brewer, 2007). The motor adaptor complex degrades in response to damage through PINK1 and parkin proteins (Wang et al., 2011). Based on our published data and preliminary data, the localization of mitochondria prior to cell division influences distribution and symmetry during metaphase and likewise affects inheritance during cytokinesis. It is not likely that mechanisms for motor dissociation are different between stem cell division and regular somatic mitosis, so the G2 positioning of mitochondria is an attractive hypothesis to direct mitochondrial inheritance.

**Conclusion**

Cell division is a well-orchestrated dance to ensure that daughter cells inherit the proper amounts of necessary organelles to function. While much of mitosis is focused on chromosomal inheritance, daughter cells require many cytoplasmic elements, mitochondria being of note. Mitochondria contain their own DNA and cannot be generated de novo. Through our studies, we have elucidated that mitochondria go through phases of passive and active inheritance, how mitochondria are inherited, what factors influence inheritance, and how mitochondrial proteins are regulated to ensure fidelity of the process. These mechanisms not only have importance during somatic cell division but likely have a role during asymmetric division as well.
Works Cited


