Regulation of Mitochondrial Dynamics during Apoptosis and the Cell Cycle

by

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Dissertation submitted in partial fulfillment of
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ABSTRACT

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Abstract

Homeostatic maintenance of cellular mitochondria requires a dynamic balance between fission and fusion, and disruptions in this balance have been implicated in multiple pathological conditions, including Charcot-Marie-Tooth, Parkinson's, and Alzheimer's diseases. Whereas deregulated fission and fusion can be detrimental to health and survival, controlled changes in morphology are important for processes like cellular division and apoptosis. Specifically, regulated mitochondrial fission occurs closely with cytochrome c release during apoptosis and upon entry into mitosis during the cell cycle. Using cell culture-based assays, microscopy, and fly genetics, we examine how changes in the mitochondrial network are mediated at the molecular level during apoptosis and the cell cycle.

First, we report that the fly protein Reaper induces mitochondrial fragmentation in mammalian cells, likely through inhibition of the mitochondrial fusion protein Mfn2. Reaper colocalizes with and binds to Mfn2 and its fly orthologue dMFN, and the colocalization of the two proteins is necessary for Reaper-induced mitochondrial fission. Moreover, the overexpression of dMFN inhibits Reaper-induced killing both in vitro and in vivo.

Our data and work in a number of experimental systems demonstrate a requirement for mitochondrial fragmentation during apoptosis that is conserved from

worms to flies to mammals. Our findings indicate that Reaper may function to inactivate mitochondrial metabolic function and/or to facilitate mitochondrial elimination during apoptosis.

Secondly, we characterize Drp1 degradation by the APC/C during mitotic exit and interphase. We provide evidence that APC/C^{Cdh1}-mediated degradation of Drp1 underlies both the morphological changes that occur during progression through the cell cycle and changes in mitochondrial metabolism during interphase. Inhibition of Cdh1-mediated Drp1 ubiquitylation and proteasomal degradation during interphase prevents the normal regrowth of mitochondrial networks after mitosis, prevents cyclin E accumulation, and alters the profile of lipid-derived metabolites. Our findings describe a novel role for APC/C^{Cdh1}-mediated Drp1 degradation in cell cycle-dependent changes in mitochondrial morphology and metabolic function and suggest that the APC/C^{Cdh1} complex may regulate the distinct bioenergetic needs of a growing cell during synthetic phases of the cell cycle.

Dedication

To Patrick, for his unwavering support and insistence that I have more fun; and to Kaitlyn, for a new perspective.

Contents

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List of Abbreviations

APC/C	Anaphase Promoting Complex/Cyclosome
CARD	Caspase recruitment domain
Cdk/CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
CMT-2A	Charcot-Marie-Tooth Type 2A
DAP	DRP-associated protein
DIAP	Drosophila Inhibitor of Apoptosis Protein
dMFN	Drosophila Mitofusin
DOA	Dominant Optic Atrophy
DRP	Dynamin-related protein
Drp1/Dlp1	Dynamin-related protein 1/Dynamin-like protein 1
ER	Endoplasmic reticulum
ETC	Electron transport chain
Fzo1p	Fuzzy onion 1 protein
GED	GTPase effector domain
HR	Heptad repeat
IAP	Inhibitor of Apoptosis Protein
IBM	IAP-binding motif

IMM	Inner mitochondrial membrane
IMS	Inner membrane space
MEF	Mouse embryonic fibroblast
Mfn	Mitofusin
MTKR	Mitotracker red
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
RHG	Reaper, Hid, Grim
RNAi	RNA interference
ROS	Reactive oxygen species
TM	Transmembrane
YMC	Yeast metabolic cycle

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dual grad school experience. Thank you to my family. Mom and Dad, your crazy dinner stories and fascination with the natural world first piqued my interest in science. Dad, maybe it was all those nature shows?! To you both and my siblings, Katie, Andrew, and Mark, thank you for your constant support and love. And finally, to Patrick and Kaitlyn, thank you for making me smile when I get home.

1. Introduction

1.1 Mitochondria: the powerhouse of the cell

Mitochondria originated from ancient bacterial ancestors and entered the early eukaryotic cell between two and three billion years ago, forging an endosymbiotic relationship essential to the evolution of eukaryotic cells (Lane, 2005; Wallace, 2005). Initially observed over 100 years ago, mitochondria were first described as a collection of individual vesicles floating freely within the cytosol (reviewed in Benard and Rossignol, 2008). Advances in electron microscopy have since led to an understanding of the complexity of the mitochondrion's internal organization. Several internal compartments exist within each mitochondrion: the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM) with its internal folds or cristae, the inner membrane space (IMS) and the interior matrix (Figure 1-1, from Frey and Mannella, 2000).

The distinct organelle structure of the mitochondrion is uniquely adapted to the task producing adenosine triphosphate (ATP), the energetic currency necessary for cellular processes. Mitochondrial metabolic function is essential for cellular bioenergetics, and key anabolic and catabolic processes occur within the mitochondria. As the sites of energy production through the process of oxidative phosphorylation (OXPHOS), mitochondria process the ultimate products of cellular catabolism to produce ATP.

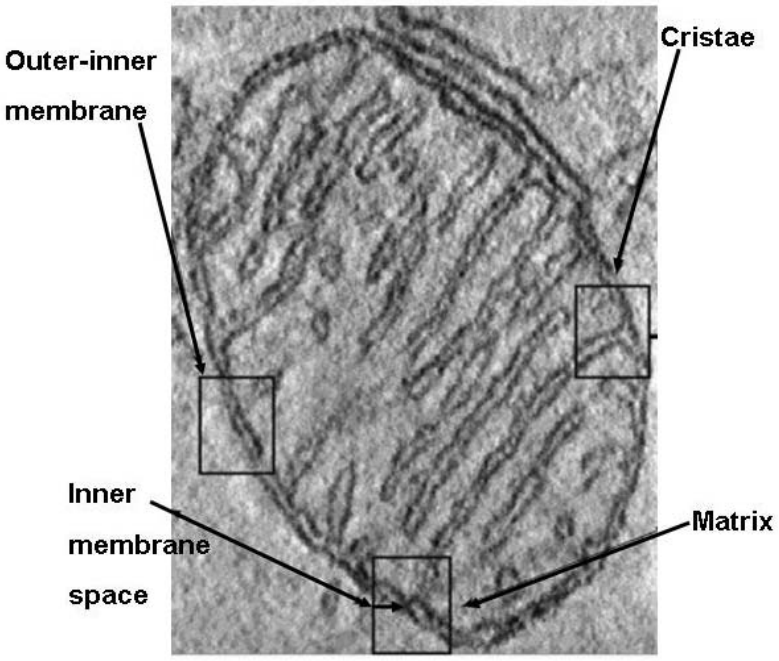


Figure 1-1: Internal structure of a mitochondrion.

A section of 3D electron microscope tomography of a single mitochondrion. From Frey and Mannella, 2000, with permission from Elsevier.

Through the oxidation of dietary carbohydrates (in the tricarboxylic acid cycle, or TCA cycle) and fats (in the process of β-oxidation), mitochondria generate the reducing equivalents NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide). These reducing equivalents are reoxidized as they pass through the electron transport chain (ETC) in the IMM, and the energy released by the flow of electrons through the respiratory chain is used to generate an electrochemical gradient across the inner mitochondrial membrane, as protons are pumped out by complexes I, III and IV of the ETC into the intermembrane space. This gradient is harnessed by the ATP synthase (complex V); the flow of protons through the ATP synthase channel is coupled to the conversion of ADP (adenosine diphosphate) to ATP, thus generating energy for cellular processes. As a by-product of normal OXPHOS, mitochondria produce most of the reactive oxygen species (ROS), which can cause damage to the cell as well as the mitochondrion itself.

1.2 The mitochondrial genome

Though the majority of mitochondrial genes have been lost or transferred to the nuclear genome during evolution, the organelle still contains its own mitochondrial DNA (mtDNA), RNA and protein synthesizing machinery (Gray et al., 1999). In contrast to the diploid nuclear genome, the human mitochondrial genome is polyploid, with several thousand copies per cell of circular double-stranded DNA molecules. Together with proteins, mtDNA molecules are organized into distinct DNA-protein complexes

called nucleoids. The inheritance mode of the mitochondrial genome DNA is also distinct from that of the nuclear genome; whereas nuclear genes follow Mendelian inheritance (for autosomes and the X chromosome), the mitochondrial genome follows an exclusively maternal pattern of inheritance. In mammals, paternal mitochondria are actively eliminated during embryogenesis (Kaneda et al., 1995; Sutovsky et al., 1999; Sutovsky et al., 2000).

The mitochondrial genome encodes a limited 37 genes: 13 polypeptide components of the ETC and the mitochondrial RNA machinery, consisting of 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). In addition to the 13 mitochondrially encoded proteins, an estimated 1500 proteins essential for proper mitochondrial function are encoded by the nuclear genome, including proteins necessary for both the maintenance of the mitochondrial genome (replication and repair) and for the expression (transcription and translation) of mitochondrial genomes (Wallace, 2005).

Mitochondrial metabolic function relies on input from both the nuclear genome and the mitochondrial genome. Due to this unique dependence on two genomes for proper organelle function, abnormalities of either genome can contribute to mitochondrial dysfunction. Because mitochondria are the vital, energy-producing components of all nucleated cells, mitochondrial dysfunction impacts many tissues, and mitochondrial disorders present with a wide range of clinical features. Classical

mitochondrial syndromes (with known mutations in the mtDNA itself) typically affect the brain, heart, skeletal muscle, kidneys, and endocrine system, though mitochondrial defects also contribute to many common diseases and disorders like diabetes, hypertension and hypercholesterolaemia (Taylor and Turnbull, 2005; Wallace, 2005).

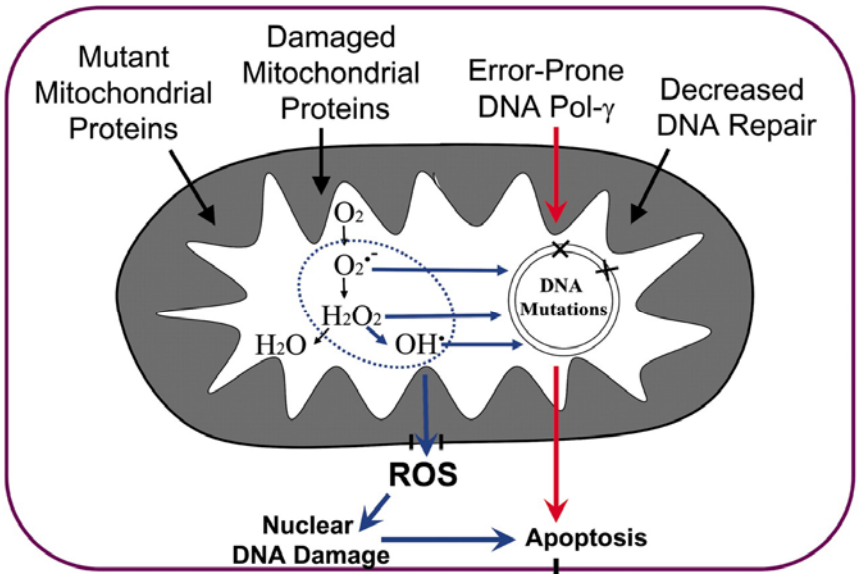
Due to its polyploid nature, the mitochondrial genome can be described as being in a state of homoplasmy (when all copies are the same) or heteroplasmy (when there is a mixture of mitochondrial genomes), and this feature of mitochondrial genetics is especially relevant in the context of pathogenic mtDNA mutations that lead to mitochondrial dysfunction and disease. Such mutations can either be homoplasmic (affecting all copies of the mitochondrial genome) or heteroplasmic (affecting only some copies). Due to the high mutation rate of mtDNA (caused in part by mitochondrial ROS), it is likely that most individuals have low levels of heteroplasmy, although there may not be any clinical evidence of this condition (such a low level of mutation might not be detectable by tissue or blood sample, and the individual might display any clinical features of mitochondrial dysfunction) (Chan, 2006; Taylor and Turnbull, 2005; Wallace, 2005).

Beyond a threshold level of mutant mtDNA, mitochondrial respiratory capacity begins to decline, ultimately resulting in cellular dysfunction. Because of their particular dependence on mitochondrial respiratory function and lower bioenergetic thresholds, tissues like the brain, heart, skeletal muscle, and endocrine glands are typically affected

in classic mitochondrial disorders (Chan, 2006). According to a theory of cellular aging based on mitochondrial function, the accumulation of mutant mtDNA over time leads to decreased energetic output, increased ROS production, and an increased propensity for programmed cell death, or apoptosis (Wallace, 2005). Ultimately, once tissue cellularity decreases beyond a functional threshold (due to decline in mitochondrial function), the clinical symptoms of age-related metabolic and degenerative disease begin to appear (Wallace, 2005) (Figure 1-2, from Loeb et al., 2005; Wallace, 2005).

The age-related worsening of clinical symptoms associated with declining mitochondrial function has been explained by the nature of mitochondrial inheritance (Chan, 2006; Dimauro and Davidzon, 2005; DiMauro and Schon, 2003; Taylor and Turnbull, 2005; Wallace, 2005). Because mitochondrial genomes are polyploid and each individual mitochondrion contains several mtDNA genomes, the possibility of genetic drift exists during cell division. Due to the random distribution of mitochondria into daughter cells during mitosis, there is a potential that one daughter cell will receive a higher ratio of mutant mtDNA (relative to wild-type mtDNA). Cells with proportionally higher levels of mutant mtDNA have a lower respiratory capacity and risk crossing below the threshold level of mitochondrial energetic output required for cellular function. If this type of drift event occurs early in the development of a tissue, an entire tissue might be harmfully affected (Chan, 2006).

A



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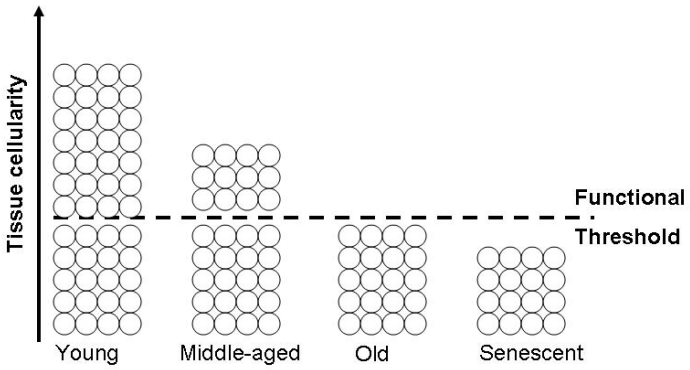


Figure 1-2: Mitochondrial theory of aging.

(A) Schematic of factors impinging upon mitochondrial integrity that can lead to loss of cellular function, cell death and aging. In blue arrows, the classical pathway is indicated: the generation of ROS as a normal by-product of OXPHOS results in damage to mitochondrial macromolecules including the mtDNA. In red arrows, the contribution of excessive mutations in mtDNA to loss of mitochondrial function is illustrated. When mitochondrial function becomes impaired beyond a threshold, apoptosis and aging can occur. From Loeb et al., 2005, with permission from PNAS. (B) Cellular model of aging. Over the life of an individual, the cumulative loss of cells due to mitochondrial cell death ultimately leads to loss of tissue function, as the cell number passes below the functional threshold. Based on Wallace, 2005.

Due to the maternal mode of inheritance for the mitochondrial genome, the potential for genetic drift in mtDNA is especially relevant during the process of oogenesis. The primordial germ cells are thought to contain very few of the founder mitochondrial genomes necessary to populate the oocytes (and ultimately the organism) (May-Panloup et al., 2007). During the process of folliculogenesis, a restriction event (or genetic bottleneck) occurs in which a random subset of mtDNA templates are selectively replicated, resulting in a reduction of total mitochondrial genetic information in the oocytes (Wai et al., 2008). This developmental bottleneck results in variation in the mtDNA that is transmitted to each oocyte (and the resulting offspring). Such variability makes it difficult to predict the extent to which an individual offspring might be affected by an inherited mtDNA mutation, as offspring developing from oocytes with high levels of mutation would risk developing disease, whereas offspring with only low levels of mutation would be unaffected (Taylor and Turnbull, 2005).

1.3 Mitochondria: dynamic organelles

Though the possibility for genetic drift within the mitochondrial genomes exists, this limited view of mtDNA inheritance does not account for the mitigating effects of mitochondrial dynamics. Mitochondria are not distinct particles, but instead form a highly dynamic reticulum; a single mitochondrion undergoes frequent fusion and fission events. Fusion with neighboring mitochondria results in the mixing of both the inner and outer mitochondrial membranes as well as the contents of the mitochondrial

matrix. Such mixing of contents between mitochondria is thought to provide a protective buffer against a potentially deleterious increase in the ratio of mutant to wild-type mtDNA (Chan, 2006). Significant experimental evidence supports both a role for mitochondrial dynamics in the complementation of mtDNA gene products and a requirement for mitochondrial dynamics in maintaining the respiratory output necessary to support cellular demands (Chen et al., 2007a; Legros et al., 2004; Nakada et al., 2001; Nunnari et al., 1997; Ono et al., 2001) (Figure 1-3, from Chan, 2006).

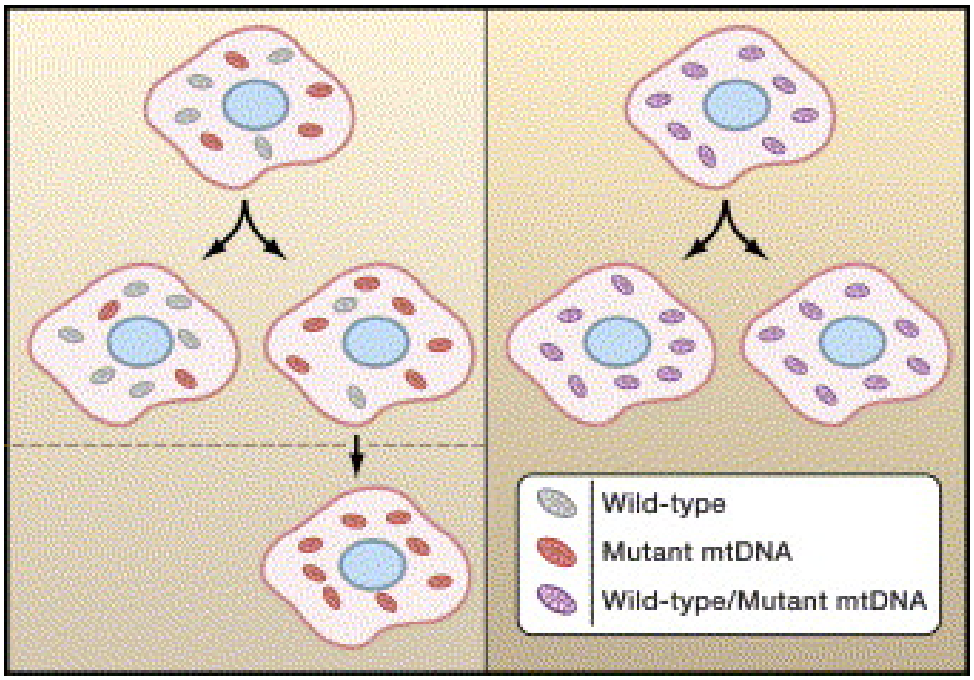


Figure 1-3: A dynamic view of mitochondrial DNA inheritance.

In the left panel, the traditional view of mtDNA inheritance is shown. In this view, mitochondria are considered to be disrete particles. During cell division, random segregation can give rise to a daughter cell with a high mutational load, and subsequent divisions can ultimately result in homoplasmy. In the right panel, the model of mtDNA inheritance incorporates mitochondrial dynamics. Due to mitochondrial fusion, mitochondria in heteroplasmic cells contain both wild-type and mutant mtDNA. Consequently, the potential for genetic drift during cell division is minimized. From Chan, 2006, with permission from Elsevier.

1.4 The mitochondrial morphology machinery

Changes in mitochondrial dynamics (also referred to as mitochondrial morphology) are mediated by a conserved family of dynamin-related protein (DRP) GTPases, consisting of the mitochondrial fusion proteins mitofusins 1 and 2 (Mfn1 and Mfn2) and Opa1, and the mitochondrial fission protein dynamin-related protein 1 (Drp1). Mutational analyses in budding yeast, *Saccharomyces cerevisiae*, and the fly, *Drosophila melanogaster*, provided the earliest molecular understanding of the fission and fusion processes, and key components of the fission/fusion machinery are well conserved in higher eukaryotes (Figure 1-4, from Cerveny et al., 2007).

1.4.1 Mitochondrial fusion

The mammalian mitofusins Mfn1 and Mfn2 are required for fusion of the outer mitochondrial membrane, and this family of proteins has been well conserved throughout evolution. The first family member discovered was the fly fusion protein, Fuzzy onion 1 protein (Fzo1p), initially identified for its essential role in sperm differentiation (Hales and Fuller, 1997). Like other members of the DRP family, the mitofusins are large GTPases. Both GTP binding and hydrolysis are required for their mitochondrial fusion function, though how GTPase activity contributes to membrane fusion is still not fully understood.

In addition to their N-terminal GTPase domain, the mitofusins contain two transmembrane domains (TM) in their C-terminal regions. The TM domains transverse the outer membrane twice, so that both the N-terminal and C-terminal portions of the protein face the cytosol. On either side of the TM domains, two heptad repeat regions exist (HR1 and HR2), and these regions are predicted to form coiled-coil structures important for mitofusin oligomerization and membrane fusion. The mitofusins work in trans, on adjacent mitochondria, and the proteins are capable of forming both homotypic and heterotypic complexes (Chen et al., 2003; Koshiba et al., 2004) (Figure 1-5, from Grandemange et al., 2009; Knott et al., 2008).

The physiological importance of mitochondrial fusion in both development and cellular homeostasis has been best highlighted by studying mice deficient in either Mfn1 or Mfn2. The absence of either mitofusin results in embryonic lethality. Loss of Mfn1 results in embryo loss around day 10.5, and mutant embryos are significantly smaller in size with pronounced developmental delays. Loss of Mfn2 results in embryonic loss around day 9.5, and the lethality is attributed to placental insufficiency. Specifically, Mfn2 mutant embryos show defects in the giant cell layer of the trophoblast; giant cells are decreased in both number and size (Chen et al., 2003).

Both Mfn1- and Mfn2-deficient cells display dramatic mitochondrial fragmentation due to lack of mitochondrial fusion. The overexpression of a single mitofusin can rescue mitochondrial fusion in the absence of the other, suggesting that

Mfn1 and Mfn2 can complement each other, though their functions are not completely redundant. In the absence of all mitochondrial fusion (through loss of both Mfn1 and Mfn2), cells show reduced growth rates, reduced respiratory capacity, and the loss of mitochondrial membrane potential. Such defects are consistent with a protective role for mitochondrial fusion; through the mixing of both the inner and outer mitochondrial membranes and matrix components, fusion provides a safeguard against a potentially deleterious decline in mitochondrial respiratory function (Chen et al., 2005; Chen et al., 2003).

Whereas mitofusins are required for outer mitochondrial fusion, another dynamin-related GTPase, Opa1, is required for fusion of the inner membrane. Multiple isoforms of Opa1 exist, generated both by alternative splicing as well as proteolytic processing. Opa1 localizes to the intermembrane space and is associated with the inner mitochondrial membrane. In addition to its role in mitochondrial fusion, Opa1 is also essential for maintaining mitochondrial cristae structure (Cipolat et al., 2006; Frezza et al., 2006; Meeusen et al., 2006).

1.4.1.1 Mitochondrial fusion in human disease

Opa1 is mutated in the most common form of autosomally inherited blindness, Dominant Optic Atrophy (DOA), which is characterized by the loss of the retinal ganglion cells whose axons form the optic nerve (Alexander et al., 2000; Delettre et al., 2000). Most of the pathogenic mutations in Opa1 that are associated with the disease

phenotype map to the GTPase domain of the protein, though how these mutations generate the clinical symptoms is still not fully understood (Ferre et al., 2005).

Like Opa1, mutations in the fusion protein Mfn2 result in a neurodegenerative disease, the common hereditary neuropathy Charcot-Marie-Tooth (CMT) disease. Mutations in Mfn2 are associated with the subtype CMT-2A, an axonal defect that causes the progressive loss of long peripheral nerves, resulting in distal motor and sensory impairments. Similar to Opa1, most known pathogenic mutations in Mfn2 also occur in or near the GTPase domain, and the resulting mitochondrial aggregation defects and mitochondrial transport defects contribute to the disease phenotype (Baloh et al., 2007; Chen et al., 2007a; Detmer and Chan, 2007). The neurodegenerative phenotype underscores the particular susceptibility of neurons to defects in mitochondrial dynamics and highlights the unique contribution of the fission/fusion processes to cellular function in the nervous system.

In addition to its better-characterized role in mitochondrial fusion, additional cellular roles are emerging for Mfn2. Recent work identified an anti-proliferative role for Mfn2 independent of its role in mitochondrial fusion; the expression of Mfn2 in vascular smooth muscle cells induces a cell cycle arrest at G1-S (Chen et al., 2004). Additionally, Mfn2 was recently discovered to be required on the membrane of the endoplasmic reticulum (ER), where it tethers mitochondria to the ER, and thus regulates both ER calcium levels (Ca^{2+}) and mitochondrial Ca^{2+} uptake (de Brito and Scorrano, 2008).

1.4.2 Mitochondrial fission

Mitochondrial fission opposes mitochondrial fusion, and the balance between the two processes is essential for cellular function. Whereas ongoing fusion and fission are important for homeostatic maintenance of cellular mitochondria, coordinated fission events do occur within the cell. During apoptosis and mitosis, mitochondria undergo regulated fragmentation, and these Drp1-mediated fission events are essential for the cell death and division processes.

In contrast to the mitofusins and Opa1, most of Drp1 is localized throughout the cell. When mitochondria are undergoing division, Drp1 localizes to mitochondrial fission sites (called fission foci or puncta) at the ends of mitochondria, where it forms complexes with DRP-associated proteins (DAPs) that regulate its function (Lackner et al., 2009). The tail-anchored integral membrane protein Fis1 is the best-characterized mammalian DAP, and it is required for proper mitochondrial targeting of Drp1 and the formation of fission foci (Mozdy et al., 2000).

Like the other DRP family members, Drp1 is a GTPase, and GTP hydrolysis by Drp1 is required for mitochondrial fission. Drp1 contains a GTPase domain at its N-terminus, a middle domain necessary for dimer formation, and a C-terminal GTPase effector domain (GED), which forms both intra- and inter-molecular interactions with Drp1 molecules to stimulate its GTPase activity (Hoppins et al., 2007). In fission foci, Drp1 binding to GTP drives its self-assembly into high-order oligomers that form helical

structures capable of constricting the mitochondrial membrane (Hoppins et al., 2007; Ingerman et al., 2005). Both the assembly of Drp1 complexes at the membrane and the hydrolysis of GTP contribute to membrane constriction, though the exact mechanism of membrane fission is not known (Figure 1-5).

1.4.2.1 Defects in mitochondrial fission

The generation of Drp1-null mice has demonstrated its essential roles in both embryonic development and cellular function. Two recent works have reported that the loss of Drp1 during development results in embryonic lethality (between days 11.5 and 12.5), and mice lacking Drp1 have severe developmental abnormalities, particularly in the cerebellum and forebrain (Ishihara et al., 2009; Wakabayashi et al., 2009). The abnormalities observed in the absence of Drp1 highlight the importance of Drp1-dependent fission during brain development, as highly polarized cells like neurons fail to develop due to defects in distributing mitochondria within the cell processes (Ishihara et al., 2009; Wakabayashi et al., 2009). In addition to the data from Drp1-null mice, a human mutation in Drp1 was recently reported. This dominant-negative mutation resulted in lethality at post-natal day 37, and the newborn had a broad-range of developmental and metabolic aberrations (Waterham et al., 2007). Whereas the human mutations in Opa1 and Mfn2 cause the neuropathies DOA and CMT-2A, respectively, the patient with a Drp1 mutation suffered severe, early-onset defects arising from lack of

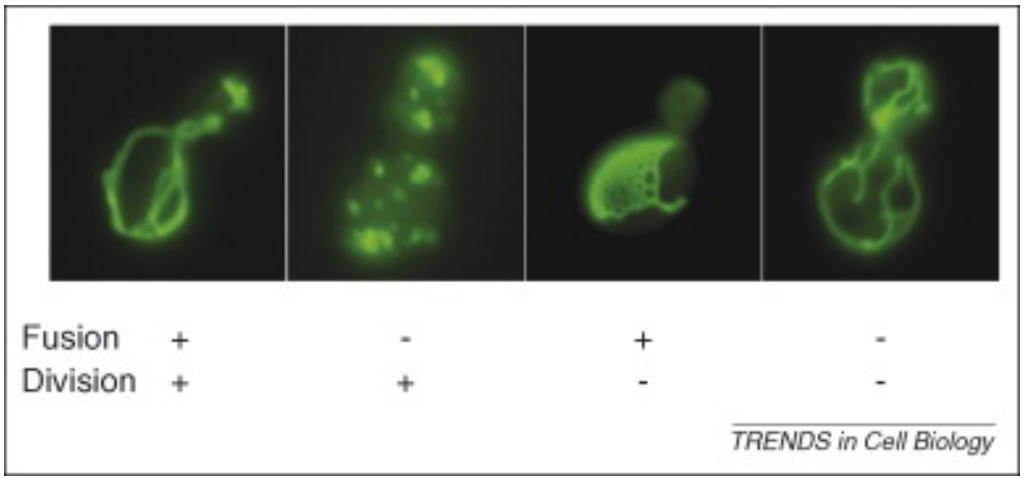


Figure 1-4: Mutations in the mitochondrial fission and fusion machinery alter mitochondrial morphology.

Morphology of yeast mitochondria in wild-type cells and in mutants defective for mitochondrial fusion, division, or both. As demonstrated by the double mutant (defective in both fusion and division), the fusion and fission processes act antagonistically to determine mitochondrial shape. From Cerveny et al., 2007, with permission from Elsevier.

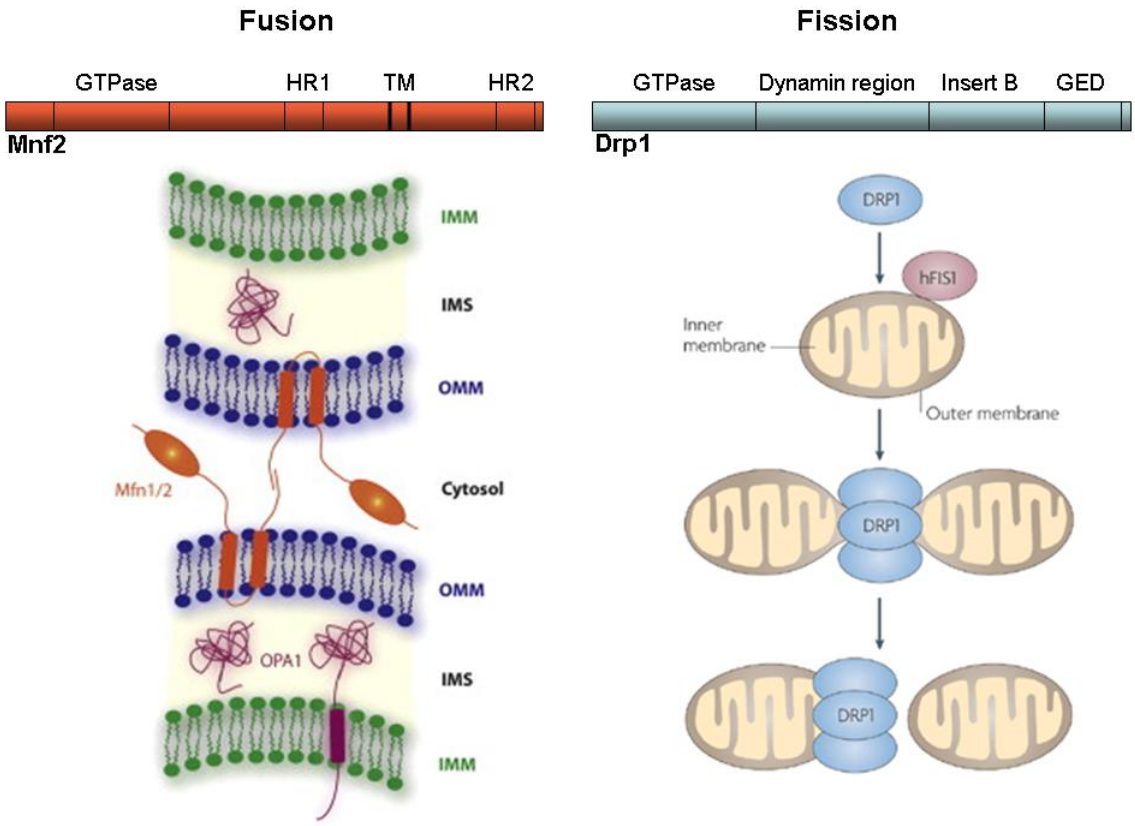


Figure 1-5: Models of mitochondrial fusion and fission.

In the left panel, a schematic of Mfn2 protein structure is shown, with its N-terminal GTPase domain, heptad repeat (HR) regions 1 and 2, and the transmembrane domain (TM). The mitofusins tether adjacent outer mitochondrial membranes and mediate outer membrane fusion, whereas Opa1 mediates fusion of the inner membrane. Adapted from Grandemange et al., 2009, with permission from Elsevier. In the right panel, a schematic of Drp1 protein is shown, with its N-terminal GTPase domain, middle domain, and C-terminal GTPase Effector Domain (GED). Drp1 is recruited to the mitochondrial surface through interaction with Fis1, where it forms foci that mediate mitochondrial fission. Adapted from Knott et al., 2008, with permission from Nature Publishing Group.

mitochondrial fission, highlighting the essential role of Drp1-mediated fission in human health.

Changes in mitochondrial morphology may influence the utilization of different substrates for energy production, and there is growing evidence to suggest that mitochondrial shape may also affect the efficiency of energy production (Benard et al., 2007; Benard and Rossignol, 2008; Jakobs et al., 2003; Meeusen et al., 2004). Experimental evidence suggests a correlation between mitochondrial network status and the type of cellular energy substrate utilized, with oxidative substrates yielding a more thin, branched mitochondrial network (that is more efficient in mitochondrial fusion) than glycolytic substrates (Benard et al., 2007; Benard and Rossignol, 2008; Jakobs et al., 2003; Meeusen et al., 2004; Rossignol et al., 2004). Mitochondrial defects such as those occurring in obesity and insulin resistance are associated with decreased Mfn2 expression and mitochondrial network formation (Zorzano, 2009). Mitochondria fragment when exposed to high glucose and fat levels in cell models of type 2 diabetes, and inhibition of Drp1-induced mitochondrial fission has been shown to ameliorate high glucose-mediated cell death (Molina et al., 2009; Yu et al., 2006a). Together with the metabolic abnormalities observed in the patient with a Drp1 mutation, these observations reveal an important relationship between mitochondrial network status and metabolic efficiency.

In addition to the growing link between mitochondrial network formation and metabolism, another role is emerging for the fission and fusion machinery in mitochondrial quality control. Genetic data from *Drosophila melanogaster* indicate that key players in mitochondrial fission and fusion are engaged in pathways which eliminate dysfunctional mitochondria, including the selective removal of damaged mitochondria by the E3 ubiquitin ligase Parkin, a gene mutated in Parkinson's disease (Deng et al., 2008; Narendra et al., 2008; Poole et al., 2008). The fission and fusion machinery is also involved in preventing mitochondria that have low membrane potential from engaging in fusion events and in facilitating their removal through a form of autophagy known as mitophagy (Twig et al., 2008).

1.4.3 Regulation of the fusion and fission machinery: post-translational modifications

Due to the important relationship between mitochondrial dynamics and mitochondrial function, it is not surprising that proteins involved in mitochondrial fission and fusion are under tight regulation, receiving inputs from various signaling pathways to meet changing cellular needs. Phosphorylation, sumoylation, nitrosylation, and ubiquitylation have been reported to affect various aspects of Drp1 function, including its localization, stability, and GTPase activity (Chang and Blackstone, 2007; Cho et al., 2009; Cribbs and Strack, 2007; Han et al., 2008; Harder et al., 2004; Karbowski et al., 2007; Taguchi et al., 2007; Wasiak et al., 2007; Zunino et al., 2009; Zunino et al., 2007). The yeast mitofusin homologue Fzo1p undergoes proteasomal degradation, and

increasing evidence supports a role for mammalian E3 ubiquitin ligases in the regulation of mitochondrial fusion (Escobar-Henriques et al., 2006; Fritz et al., 2003; Nakamura et al., 2006; Neutzner et al., 2008; Park et al.; Yonashiro et al., 2006). The complex regulation of the mitochondrial fission and fusion machinery by post-translational modifications provides a means of fine-tuning fission and fusion events to suit cellular demands, and it is further evidence of cross-talk between cellular signaling and mitochondrial signaling pathways.

1.5 Regulated changes in mitochondrial morphology

1.5.1 Apoptotic mitochondrial fission

Mitochondria provide the cellular setting for key signaling events of programmed cell death, or apoptosis. The localization of key apoptotic proteins to the mitochondria has been conserved throughout evolution, and in mammals, release of respiratory chain component cytochrome c from the IMS of the mitochondria initiates the downstream events of the apoptosis. Dramatic changes in mitochondrial morphology occur during apoptosis; mitochondria undergo Drp1-dependent fission early in the cell death pathway (Desagher and Martinou, 2000; Frank et al., 2001; Mancini et al., 1997; Youle and Karbowski, 2005). These morphological changes are also evolutionarily conserved; in the fly, the worm *Caenorhabditis elegans,* and the mouse, mitochondrial fission is necessary for the proper execution of the apoptotic program

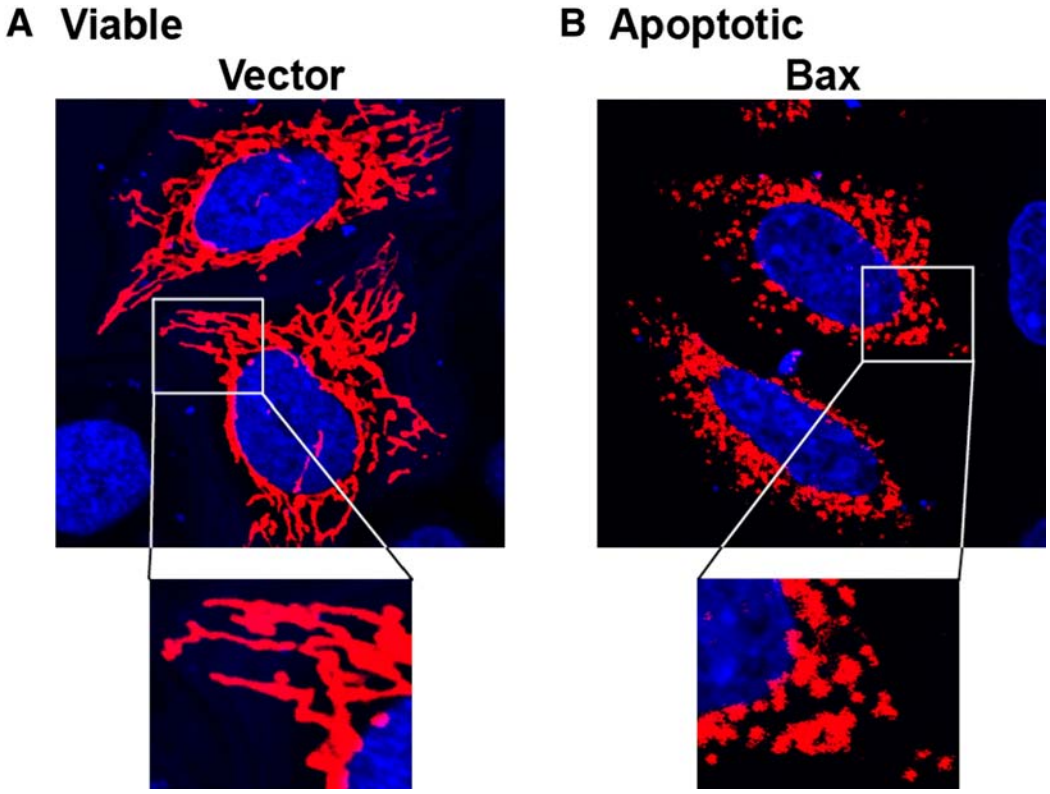


Figure 1-6: Mitochondrial fission during apoptosis.

Hela cells were transfected with mito-RFP to visualize mitochondrial networks, together with either an empty vector plasmid or a plasmid expressing the pro-apoptotic Bcl-2 family member Bax. From Autret and Martin, 2009, with permission from Elsevier.

(Abdelwahid et al., 2007; Goyal et al., 2007; Jagasia et al., 2005; Wakabayashi et al., 2009) (Figure 1-6, from Autret and Martin, 2009).

1.5.1.1 The apoptotic pathway

Apoptosis is a regulated process by which a cell dismantles itself, and it is critical for tissue homeostasis and remodeling during development. Apoptosis can be initiated in response to various stimuli, such as DNA damage, death ligand binding to extracellular receptors, and developmental cues. In contrast to necrosis, an apoptotic death prevents inflammation and damage to the surrounding tissue by targeting the dying cell for phagocytosis by macrophages. The dying cell is characterized by a series of morphological changes including cell shrinkage, DNA fragmentation, and blebbing of the plasma membrane. Molecularly, these changes are mediated by caspases, a family of aspartate-directed cysteine proteases. Synthesized as inactive zymogens, caspases are activated in a complex cleavage cascade in response to various apoptotic stimuli. Upstream caspases called initiator or apical caspases are capable of autocatalytic activation, facilitated by adaptor proteins. Downstream or effector caspases are cleaved and activated by initiator caspases.

In vertebrate cells, the primary control point for caspase activation is the release of cytochrome c from mitochondria, which is regulated by members of the Bcl-2 protein family. In contrast, the main point of control for apoptosis in *Drosophila melanogaster* resides with the Drosophila Inhibitor of Apoptosis (DIAP) proteins, which suppress

active caspases prior to activation of the apoptotic program. DIAP can be antagonized by the proteins Reaper, Hid and Grim (RHG), which physically displace DIAPs from caspases and also induce DIAP degradation (Bergmann et al., 2003). Through these activities, the RHG proteins act as major inducers of fly apoptosis.

In the intrinsic apoptotic pathway, the release of cytochrome c from the mitochondrial inter-membrane space triggers caspase activation (Liu et al., 1996). In the presence of dATP, cytosolic cytochrome c binds the adaptor protein Apaf-1, exposing its caspase recruitment domain (CARD), which recruits the initiator caspase-9 into a large complex known as the apoptosome (Li et al., 1997). The apoptosome-bound caspase-9 cleaves and activates the main effector caspase, caspase-3. Activation of caspase-3 and other effector caspases produces the morphological hallmarks of apoptosis (Danial and Korsmeyer, 2004).

Both *C. elegans* and *D. melanogaster* have Apaf-1 homologues that function to mediate caspase activation. The worm homologue, CED-4, has a CARD domain like Apaf-1, but does not require cytochrome c binding for activation and is regulated directly by CED-9, the *C. elegans* anti-apoptotic Bcl-2 family member. The fly homologue, DARK, has both the CARD domain and the WD40 domains to which cytochrome c binds in the vertebrate pathway. However, in the fly, the mechanism of DARK regulation is unclear, and many cells experience constitutive activation of the apical caspase Dronc, which is suppressed by the DIAP proteins (Muro et al., 2002).

Members of the Bcl-2 protein family both positively and negatively regulate apoptosis. Anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-xL, contain four Bcl-2 homology (BH1-4) domains and antagonize the function of pro-apoptotic family members via heterodimerization to block mitochondrial cytochrome c release. Pro-apoptotic members of this family are further subdivided into the "multidomain" members like Bax, Bak, and Bok, which possess BH1-3 domains, and the "BH3-only" members such as Bid and Bad.

Mice lacking both Bax and Bak are resistant to multiple apoptotic stimuli that act through disruption of mitochondrial function, suggesting that activation of these multi-domain members is an essential step for cell death in the mitochondrial pathway (Wei et al., 2001). Anti-apoptotic members like Bcl-2 and Bcl-xL heterodimerize with Bax and Bak at the mitochondria, preventing cytochrome c release and caspase activation. Additionally, BH-3 only proteins act pro-apoptotically by directly activating Bax or Bak and/or by inhibiting the anti-apoptotic members (Adams and Cory, 1998; Danial and Korsmeyer, 2004). Because the primary control point for caspase activation in vertebrate cells is the release of cytochrome c, the Bcl-2 protein family constitutes a major point of apoptotic regulation. In contrast to regulating cytochrome c release, Bcl-2 family members in *C. elegans* function to regulate the Apaf-1 homologue CED-4 directly. In the fly, Bcl-2 proteins play only a limited role in the regulation apoptosis, and their

mechanism of action is not fully understood (Colussi et al., 2000; Galindo et al., 2009; Mollereau, 2009).

Once activated, caspase function can be neutralized through the binding of Inhibitor of Apoptosis (IAP), though the extent to which IAP proteins function to block apoptosis appears to vary in different organisms and cell types. Despite its important anti-apoptotic function, XIAP deficient mice are viable and have no detectable defects in caspase dependent or independent death (Harlin et al., 2001). In contrast, in non-developmental cell death, IAPs have been shown to stringently regulate apoptosis in terminally differentiated neuronal cells (Wright et al., 2004).

In *D. melanogaster*, as in differentiated neurons, regulation of IAPs is an important point of apoptotic regulation. The removal of DIAP1 from fly cells or embryos is sufficient to induce rapid apoptosis, suggesting that caspases are already active and are only inhibited by IAP binding (Hay et al., 1995; Lisi et al., 2000; Muro et al., 2002; Yoo et al., 2002). In contrast to the vertebrate pathway, the levels of IAP proteins appear to constitute the main control point for fly apoptosis.

DIAP1 levels constitute the main regulatory point for fly apoptosis, and the key inducers of apoptosis, Reaper, Hid and Grim, act through antagonism of DIAP proteins. A chromosomal deletion (the H99 deletion) that removes the genes *reaper*, *hid*, and *grim* (RHG) inhibits cell death during development and in response to cytotoxic γ-irradiation (White et al., 1994). The Reaper, Hid and Grim proteins share an N-terminal IAP-

binding motif (IBM) and bind IAPs in the same BIR2 domain as active caspases, effectively displacing caspases and liberating them from IAP-inhibition. Although no close vertebrate homologues of the RHG proteins have yet been reported, there are two mammalian proteins which share the IBM domain. Smac/DIABLO and Omi/HtrA2 have been shown to bind and inhibit mammalian IAPs through their IBM domains, but these proteins do not share any homology with RHG proteins outside of the IBM (Saelens et al., 2004). Whereas the RHG have been shown to localize to the outer surface of the mitochondria, the Smac/DIABLO and Omi/HtrA2 proteins are located within mitochondria and are released during apoptosis (Du et al., 2000; Haining et al., 1999).

Although mitochondrial cytochrome c release does not mediate fly apoptosis (except for in specific cell types), both the Reaper and Grim proteins can potently trigger cytochrome c release and caspase activation when expressed in mammalian cells and vertebrate cell-free lysates (Claveria et al., 1998; Evans et al., 1997). This ability suggests that these proteins have the capacity to act on mitochondria. Indeed, the mitochondrial localization of the RHG proteins is required for their full pro-apoptotic activity (Abdelwahid et al., 2007; Claveria et al., 2002; Haining et al., 1999; Olson et al., 2003).

1.5.1.2 Interactions between the cell death machinery and the fission/fusion machinery

The contribution Drp1-mediated fission to progression of the apoptotic program appears to vary depending on cell type and apoptotic stimulus. During worm, fly and

mouse development and in some mammalian cell culture-based assays, inhibiting Drp1 function (through genetic deletion or mutation, the use of a dominant- negative Drp1 construct, a chemical inhibitor of Drp1, or RNA interference of Drp1 message levels) significantly decreases cellular apoptosis (Abdelwahid et al., 2007; Cassidy-Stone et al., 2008; Frank et al., 2001; Goyal et al., 2007; Jagasia et al., 2005; Lee et al., 2004; Wakabayashi et al., 2009). In contrast, other groups have reported that interfering with Drp1-dependent fusion delays (but does not prevent) cytochrome c release and caspase activation and that Drp1 functions downstream of caspase activation to facilitate mitochondrial elimination though mitophagy (Breckenridge et al., 2008; Ishihara et al., 2009).

Like Drp1, the fusion protein Opa1 has an essential role in apoptosis. BH3-only proteins and Bax and Bak induce Opa1-mediated cristae remodeling, and this apoptotic remodeling is required for cytochrome c release and apoptosis. The activity of the other mitochondrial fusion proteins, the mitofusins, has also been shown to modulate cellular responses to apoptotic stimuli; in some experimental systems, blocking mitochondrial fission by overexpression of Mfn2 interferes with Bax and Bak activation and blocks cell death (Jahani-Asl et al., 2007; Neuspiel et al., 2005; Sugioka et al., 2004).

During apoptosis, the fission protein Drp1 and the fusion protein Mfn2 colocalize with the Bcl-2 proteins Bax and Bak in fission foci prior to caspase activation (Karbowski et al., 2002; Nechushtan et al., 2001; Valentijn et al., 2003), and this interaction between

the fission/fusion machinery and the Bcl-2 protein family is not limited to mitochondrial fragmentation during apoptosis. Growing evidence supports a role for members of the Bcl-2 protein family in regulating mitochondrial dynamics, perhaps independently of their apoptotic signaling functions. Bax and Bak are required for normal mitochondrial fusion through their interactions with Mfn2, as cells lacking both Bak and Bax display fragmented mitochondria due the abnormal submitochondrial distribution of Mfn2. (Karbowski et al., 2006). Anti-apoptotic Bcl-2 family members like Bcl-xL have also been implicated in the regulation of mitochondrial dynamics. Expression of Bcl-xL increases rates of mitochondrial fission and fusion in healthy neurons, and the *C. elegans* homologue, CED-9, interacts with Mfn2 to promote fusion of the mitochondrial network (Berman et al., 2009; Delivani et al., 2006).

1.5.2 Mitochondrial fission during mitotic entry

Just as apoptosis is characterized by predominantly punctate, fragmented mitochondria, regulated mitochondrial fission also occurs during the cell cycle. Failure of Drp1-mediated fission leads to defects in cell cycle completion and in the distribution of mitochondria between daughter cells. When the mitochondrial fission machinery is inhibited, cells experience defects in cytokinesis, and mitochondria are unequally partitioned between daughter cells (Ishihara et al., 2009; Labrousse et al., 1999; Taguchi et al., 2007). The requirement for mitochondrial fragmentation during mitosis suggests that there is cross-talk between cell cycle proteins and the regulation of mitochondrial

fission during mitosis, and experimental evidence is now emerging to support this interplay (discussed in detail in Chapters 4 and 5).

1.5.2.1 The cell cycle

The cell cycle is the process by which the cell duplicates itself. It is composed of two main phases: interphase and mitosis. Interphase is further subdivided into G1, S, and G2 phases. G1 and G2 are "gap" phases in which the cell grows, and they are separated by S phase (named for the synthesis of DNA), in which the chromosomes are duplicated in preparation for division.

Mitosis is the division of the nucleus, and it is further subdivided into five phases: prophase, prometaphase, metaphase, anaphase and telophase. Mitosis is followed immediately by cytokinesis, the cytoplasmic division. During prophase, the microtubule organizing centers or centrosomes move to opposite sides of the cell and the mitotic spindle begins to form. Next, during prometaphase, the nuclear envelope breaks down, and the spindle fibers begin to attach to the kinetochores, protein structures at the centromere regions of the sister chromatids. In metaphase, the chromosomes align at the metaphase plate between the two poles of the cell, and they are held there until all kinetochores are properly attached to the mitotic spindle. Following metaphase, sister chomatid separation occurs, initiated by the APC/C (Anaphase Promoting Complex/Cyclosome). The breakdown of cohesins, the proteins that hold the kinetochores together, enables the separation of sister chromatids, and this

breakdown is mediated by separase, which is inhibited through its interaction with the protein securin. The APC/C stimulates sister chromatid separation by targeting securin for degradation, thus liberating separase to cleave cohesins. During telophase, the nuclear envelopes reform, and cytokinesis occurs, dividing the remaining cellular components into the two, genetically identical daughter cells.

Progression through the cell cycle is driven by cyclin-dependent kinases (CDKs), which phosphorylate and activate many key cell cycle proteins. CDKs are active in complex with their partner cyclins, originally named for the periodic oscillations in their levels throughout the cell cycle (Evans et al., 1983). Together with CDK-mediated phosphorylation of target proteins, the ubiquitin-mediated degradation of cyclins, cyclin-dependent kinase inhibitors (CKIs), and other cell cycle regulatory proteins ensures that progression through the cell cycle occurs in one direction. Uni-directional progression through the cell cycle coordinates each round of DNA replication with cellular division, providing a guard against genomic instability and cellular aneuploidy. The cell cycle is frequently dysregulated during oncogensis, and aberrant cell growth and division contribute to the hallmarks of cancer, as defined by Drs. Hanahan and Weinberg in their landmark review of the succession of genetic alterations that occur during cancer development (Hanahan and Weinberg, 2000).

Degradation of target proteins by the 26S proteasome during the cell cycle is initiated by the covalent attachment of ubiquitin chains to the substrates, marking them

for destruction. This step is catalyzed by at least three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). Two E3 ubiquitin ligases control the degradation of many cell cycle regulatory proteins: the SCF (Skp1/CUL1/F-box protein), which is active throughout the cell cycle, and the APC/C, which is active from mitosis through the subsequent G1 phase (Nakayama and Nakayama, 2006; Reed, 2003).

The APC/C is active in complex with one of two co-activator proteins, Cdc20 or Cdh1. APC/C^{Cdc20} is active early in mitosis, and this complex targets proteins like securin and cyclin B, whose necessary degradation initiates sister chromatid separation and the onset of anaphase. Following APC/C^{Cdc20} activity, the APC/C is next active through its association with Cdh1, and the APC/C^{Cdh1} complex remains active from anaphase of mitosis through the subsequent G1 phase.

Whereas APC/C^{Cdc20} function is required for mitotic exit, APC/C^{Cdh1} is not. Instead, APC/C^{Cdh1} is emerging as a "master regulator" of G1 and the quiescent G0 phases (Skaar and Pagano, 2008). Inhibition of Cdh1 function (by genetic deletion or using RNA interference, RNAi) demonstrates its essential role in regulating the timing of G1 phase; cells lacking Cdh1 enter S phase prematurely, but progress through S phase more slowly. Due to the improper S phase entry (and premature DNA replication), mouse embryonic fibroblasts (MEFs) null for Cdh1 display a high degree of genomic instability, supporting its role as a tumor suppressor (Garcia-Higuera et al., 2008; Li et

al., 2008). Mice lacking Cdh1 are not viable, and embryonic lethality is observed between 9.5 and 10.5 days. Though placental insufficiency is the primary cause of lethality in the homozygous null mice, the heterozygous mice display severe defects in behavior and learning due to cell cycle aberrations in the nervous system (Garcia-Higuera et al., 2008; Li et al., 2008). These defects highlight an essential role for the APC/C^{Cdh1} complex in regulating cell cycle progression through the degradation of target proteins.

1.5.2.2 Changes in mitochondrial morphology and metabolism during the cell cycle

Just as cell cycle regulators like the APC/C coordinate the division of the chromosomes during the cell cycle, these key components of the cell cycle machinery also mediate the division of the cell's cytoplasmic contents, including organelles like the mitochondria. Drp1-mediated mitochondrial fission is essential for the completion of cytokinesis and for the proper distribution of mitochondria into the daughter cells. Mitochondrial fission during mitosis is regulated by phosphorylation of Drp1 by the Cdk1/cyclin B complex (Labrousse et al., 1999; Taguchi et al., 2007). This phosphorylation is essential for mitotic division of mitochondria, as the expression of a mutant Drp1 that cannot be phosphorylated results in the unequal transmission of mitochondria to daughter cells (Taguchi et al., 2007). This interplay, in which a known cell cycle regulator (the Cdk1/cyclin B complex) induces mitochondrial fission is just one example of a bi-directional relationship between cell cycle progression and changes in mitochondrial dynamics.

Understanding cell cycle-dependent changes in the mitochondrial network has been facilitated by advances in microscopy that enable the observation of mitochondrial dynamics in living cells undergoing mitosis. Mitochondria in interphase cells have been described as an interconnected network that begins to fragment as the cells enter mitosis (Barni et al., 1996; Margineantu et al., 2002; Taguchi et al., 2007). During telophase and cytokinesis of mitotic exit, re-establishment of the network (and mitochondrial elongation) occurs, and interphase mitochondria once again form an extensive network (Mitra et al., 2009; Taguchi et al., 2007) (Figure 1-7, from Taguchi et al., 2007).

The changes in mitochondrial morphology that occur during the cell cycle are accompanied by shifts in metabolism. The yeast metabolic cycle (YMC) is coordinated with the cell cycle, in part to restrict oxidative processes from occurring during DNA replication (thus preserving genome integrity by protecting DNA from oxidative damage), and evidence supports this type of temporal compartmentalization of metabolic processes in mammalian cells as well (Chen et al., 2007b; Tu et al., 2005; Yu et al., 2009).

Significant increases in mitochondrial membrane potential and respiration occur during progression through G1 (Schieke et al., 2008). These G1-dependent changes in mitochondrial metabolism during G1 may reflect coordination between mitochondrial metabolic supply and the demands of a growing cell. Also, there is evidence that cell-cycle dependent mitochondrial biogenesis occurs, and it may provide a means of

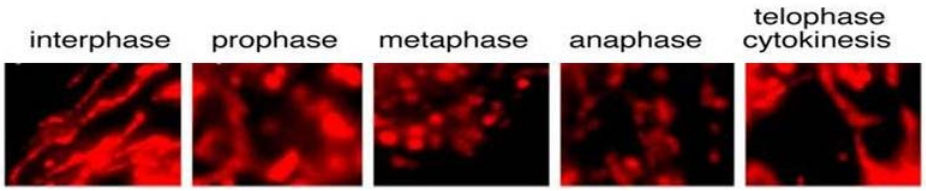


Figure 1-7: Mitochondrial morphology changes during the cell cycle.

Hela cells were transfected with su9-RFP to visualize mitochondria and synchronized prior to mitosis using thymidine. Cells were analyzed by confocal microscopy, and compiled confocal section images are shown. Adapted from Taguchi et al., 2007, with permission from *J. Biol. Chem*. This research was originally published in the *Journal of Biochemistry*. Taguchi N., Ishihara, N., Jofuku, A., Oka, T., and Mihara, K. Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. *J. Biol. Chem*., 2007, **282**:11521-11529. © The American Society for Biochemistry and Molecular Biology

integrating the expression of the respiratory apparatus with cell proliferation (Lee et al., 2007; Scarpulla, 2008).

The recent identification of retrograde signaling pathways from the mitochondria has uncovered a metabolic checkpoint at the G1/S boundary, highlighting the importance of ensuring that cellular proliferation is coordinated with sufficient metabolic capacity and proper mitochondrial function (Jones et al., 2005; Mandal et al., 2005; Mitra et al., 2009; Owusu-Ansah et al., 2008; Schieke et al., 2008; Wellen et al., 2009). In one such pathway, an extensive mitochondrial network regulates cyclin E accumulation at G1/S, and this "hyperfused" mitochondrial state is essential for progression into S-phase (Mitra et al., 2009). The cross-talk between regulators of mitochondrial dynamics and key cell cycle regulators ensures that mitochondrial function is tightly linked to cell growth and proliferation.

In this dissertation, we examine the regulation of mitochondrial fission during apoptosis and the cell cycle. By using Reaper, a fly inducer of apoptosis with a well-characterized ability to initiate cytochrome c release and caspase activation in vertebrate systems, we describe the inhibition of mitochondrial fusion during apoptosis that is conserved in both invertebrate and vertebrate systems. Though investigating mechanisms regulating mitochondrial fission during mitosis, we uncover the APC/C^{Cdh1}-mediated degradation of Drp1, as well as new evidence supporting a link between mitochondrial dynamics and metabolism.

2. Materials and methods

2.1 Plasmid cloning, site-directed mutagenesis, protein expression, and RNA interference

pEBB-RPR-Flag, pEBB-RPR1665-Flag and pEBB-RPRΔGH3 were generated by subcloning PCR products into the BamHI and NotI sites of pEBB-Flag. pCDNA3-Flag-RPR, pCDNA3-Flag-RPR1665 and pCDNA3 FlagRPRΔGH3 were generated by first cloning Reaper and the Reaper mutants into pENTR-3C (Invitrogen). Reaper was cloned into pCDNA3-Flag-DEST using LR Clonase II (Invitrogen) recombination. GFP-Drp1K38A and GFP-Mfn2 were generated by cloning hDrp1K38A and hMFN2 into pEGFP-C1(Clontech). pCDNA3-Mfn2 was a generous gift from Richard Youle (NINDS, Bethesda, MD), and pCDNA3-HA-Drp1K38A was a generous gift Alex van der Bliek (UCLA, CA). pAMW-dMFN, pAGW-dMFN, pTMW-dMFN were generated by first cloning dMFN into pENTR-3C and then performing a recombination reaction into pAMW (actin promoter with N-terminal Myc tag), pAGW (actin promoter with N-terminal GFP tag) and pTMW (pUAST vector with N-terminal Myc tag). pEGFP-mito was derived from pdsRED2-Mito vector (Clontech).

HA-tagged Cdh1 and Cdc20 plasmids were a generous gift of M. Pagano (NYU, NY). The Drp1 expression construct was kindly provided by A.M. van der Bliek (UCLA, CA), and was subcloned into pcDNA3 with a C-terminal flag tag. The Drp1 D-box mutant was prepared with a QuikChange Site-Directed Mutagenesis Kit (Stratagene)

using the following primer sequence: 5'gtg act tgt ctt ctt gcc aaa agg gcc cct gtt aca aat g 3'.

For protein production, Mfn2 and Drp1 were first subcloned into pENTR-3C (Invitrogen) and recombined into pDEST-17 via LR clonase II recombination (Invitrogen). Synthetic Reaper peptides were prepared as previously described (Holley et al., 2002). Recombinant Cdh1 protein was a generous gift from the Ronai Lab (Burnham Institute for Medical Research, CA).

siRNAs were purchased from Dharmacon. A non-targeting siRNA was used as a negative control (siGENOME siRNA #2), an ON-TARGET plus SMARTpool was used for Drp1, and the Cdh1 siRNA nucleotide sequence has been described previously (Bashir et al., 2004).

2.2 Antibodies

The primary antibodies used for immunoblotting and immunoprecipitations were as follows: anti-Drp1 (BD Transduction Laboratories); Phospho-Histone H3 (Cell Signaling); anti-actin, anti-HA, anti-Cdc27, anti-Cyclin E and Cyclin B1 (monoclonal and polyclonal) (from Santa Cruz Biotechnology); anti-Cdh1 (Thermo Scientific) and anti-FLAG (polyclonal, Sigma). The Mfn2 antibody was a gift from Dr. Richard Youle (NINDS, Bethesda, MD). AlexaFluor (Invitrogen) and IR-Dye (LI-COR Biosciences) secondary antibodies were used for immunoblotting with the LI-COR Imaging System,

or HRP-conjugated antibodies (from Promega and Dako) were used with an ECL-Plus detection system (Amersham Biosciences).

For immunofluorescence, cells were stained with the following primary antibodies: anti-Flag(Sigma), anti-HA(from Santa Cruz Biotechnology), anti-cytochrome c (BD Pharmingen) or anti-Drp1 (BD Transduction Laboratories). Following PBS washes, cells were next incubated with secondary antibodies including goat-anti-rabbit Alexa 488, goat-anti-mouse Alexa 594 , or goat-anti-rabbit Alexa 561 (Molecular Probes, Invitrogen).

2.3 Cell culture

Drosophila S2 cells were cultured in Schneider's Drosophila Medium with 10% heat-inactivated fetal bovine serum.

Hela, Hela S3, and HEK 293T cells were grown in DMEM with 10% fetal bovine serum. For double thymidine block experiments, Hela and Hela S3 cells were synchronized by sequential incubations with 2.5 mM thymidine and either released into medium containing 40 ng/ml nocodazole (for G1/S release experiments) or analyzed during the second thymidine block (for mitochondrial morphology and metabolomics). To generate early G1 cells for immunofluorescence, cells were examined 14.5 to 16 h following release from the second thymidine arrest. For nocodazole release experiments, Hela and Hela S3 cells were blocked in media containing 40 ng/ml for 16 to 17 h. Non-adherent cells were washed three times in PBS and released into complete media. At the

indicated times after release, adherent cells were harvested for western blotting. For nocodazole release experiments in Cdh1 MEFS, cells were synchronized using a single thymidine block (2.5 mM) and were then released into media containing 80 ng/ml nocodazole for 17 h. Cells were released and harvested as described above. In vitro mitotic exit assays were performed as previously described (Wu et al., 2009). Proteasome inhibitors were used at the following concentrations: lactacystin (Sigma) at 30 μM for 6 h; MG132 (Calbiochem) at 20 μM for 5 h; or epoxomicin (Calbiochem) at 2.5 μM for 11h.

Cdh1 wild-type and null MEFS were generously provided by the Malumbres Lab (CNIO, Madrid), and were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mM L-Glutamine.

Drosophila S2 cell transfections were performed using Cellfectin (Invitrogen) according to the manufacturer's instructions. Plasmid transfections were performed in mammalian cells using Fugene 6 (Roche) according to the manufacturer's instructions. siRNA oligonucleotides were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. For RNAi transfection/synchronization experiments, Hela cells were plated at subconfluent densities and were transfected twice with siRNA constructs, before both the first and second thymidine block.

2.4 Lysate preparations and co-precipitations

2.4.1 Cell-free Xenopus extract preparation and co-precipitations

Crude Xenopus interphase extracts were prepared as previously described (Evans et al., 1997). To precipitate endogenous Xenopus Mfn2, Rpr16-65 peptide (32 μM) was conjugated to streptavidin-sepharose beads (Amersham) and then Reaper-conjugated beads and streptavidin-sepharose beads alone were blocked in free biotin (32 μM) prior to incubation with Xenopus egg extracts.

For preparation of His-Mfn2, pDEST17-Mfn2 was transformed into BL21-AI cells (Invitrogen), and His-Mfn2 was purified using Ni-affinity chromatography (Ni-NTA agarose, Quiagen). Ni-bound His-Mfn2 beads were washed first in Egg Lysis Buffer (ELB: 250 mM sucrose, 2.5 mM $MgCl_2$, 10 mM Hepes pH 7.7, 50 mM KCl and 1 mM DTT). Control nickel beards were pre-blocked in 0.125 M Histidine, and then both sets of bead (control nickel and Ni-bound His-Mfn2) were blocked in 1% casamino acids and then washed in ELB prior to incubation with Reaper peptide. Control Ni-beads and Ni-bound His-Mfn2 beads were incubated in Xenopus egg extracts treated with DMSO control or Reaper peptide (16 μM). Following 1 h incubation at 4º C, the beads were washed three times in ELB prior to SDS-PAGE and immunoblotting.

Rpr16-65 peptide (32 μM) was conjugated to streptavidin-sepharose beads (Amersham) and then Reaper-conjugated beads and streptavidin-sepharose beads alone were blocked in free biotin (32 μM) prior to incubation with the cell lysates.

The APC assay was performed as previously described (Wu et al., 2007) with the following modifications: immunoprecipitated Xenopus APC was incubated with either Cdc20 or Cdh1 at room temperature for 1 hr, and then ^{35}S-labeled human Drp1 was added in the presence of recombinant E1, E2, and ATP.

2.4.2 Cell lysate preparation and co-precipitations

2.4.2.1 Drosophila S2 cell lysate preparation and co-precipitations

Drosophila S2 cells were resuspended in hypotonic buffer (20 mM Hepes-KOH pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM Sucrose, 1 mM DTT and with Complete Protease Inhibitors (Roche) and allowed to swell on ice 1 h prior to lysis by sonication. Rpr16-65 peptide (32 μM) was conjugated to streptavidin-sepharose beads (Amersham) and then Reaper-conjugated beads and streptavidin-sepharose beads alone were blocked in free biotin (32 μM) prior to incubation with the cell lysates. Cleared cell lysates were incubated with beads for 2 h at 4º C . Beads were then washed three times in PBS with 300 mM NaCl before loading onto SDS-PAGE gels for immunoblotting.

2.4.2.1 Mammalian cell lysate preparation and co-precipitations

For western blotting, cells were lysed in cold RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1mM EDTA, 0.1% SDS, 150 mM NaCl supplemented with 10 ug/ml aprotonin and leupeptin and 1 mM sodium vanadate).

Lysate concentrations were determined by Bradford Assay (Biorad), and concentrations were normalized before loading onto SDS-PAGE gels.

For immunoprecipitation experiments, 293T cells were lysed with IP Buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and Complete Protease Inhibitors (Roche)), and extracts were incubated with HA antibody (3 μg) for 1 h at 4º C before Protein A Sepharose was added. Immunoprecipitates were washed three times in IP Buffer before loading onto SDS-PAGE gels for immunoblotting.

For Drp1-ubiquitylation experiments, wild-type or Cdh1 null MEFS were pretreated with 20 μM MG132 for 6 h prior to lysis in IP Buffer. Lysates were quantitated by Bradford Assay (Biorad), and protein concentrations were normalized prior to incubation with Drp1-conjugated Protein G sepharose (Sigma) for 2 h at 4º C. Immunoprecipitates were washed three times in IP Buffer prior to SDS-PAGE and immunoblotting.

For His-Drp1 pulldowns, pDEST17-Drp1 was transformed into BL21-AI cells (Invitrogen), and His-Drp1 was purified using Ni-affinity chromatography (Ni-NTA agarose, Quiagen). Ni-bound His-Drp1 beads were washed first in wash buffer containing 10 mM Hepes pH 7.5, 500 mM NaCl with 0.02% Triton X-100 and then in wash buffer without Triton X-100. Control nickel beards were pre-blocked in 0.125 M Histidine. Both sets of bead (control nickel and Ni-bound His-Drp1) were washed in IP buffer and then incubated with Hela cell lysates (lysed in IP Buffer) for 2 h at 4º C. The

beads were washed three times in IP buffer before loading onto SDS-PAGE gels for immunoblotting.

2.5 Immunofluorescence and cell imaging

Prior to immunostaining and imaging, S2 cells were fixed in suspension with 4% formaldehyde and permeabilized with 2.5% Triton X-100.

Imaging of Hela cells was performed by plating Hela cells at sub-confluent densities on Matek coverslip chamber slides or glass-bottom dishes (MatTek Corporation). Based on our observations of mitochondrial morphology in Hela cells exiting mitosis, we noted a discrepancy in the timing of mitochondrial elongation and network establishment between cells exiting release from a nocodazole arrest and cells that had been arrested at the G1/S border and allowed to progress through mitosis in absence of nocodazole. Specifically, nocodazole-arrested cells did not show mitochondrial network formation until six hours following release, whereas reformation of the network occurred very quickly during mitotic exit in our experiments using unperturbed live cells, consistent with previous reports. This discrepancy can likely be attributed to the importance of microtubules in maintaining mitochondrial morphology (Frederick and Shaw, 2007). Thus, for synchronized cell experiments, we examined mitochondrial morphology either at the G1/S boundary or 14.5 h following release from double thymidine arrest (early G1) instead.

Cells were fixed in 4% formaldehyde for 10 minutes, permeabilized with 0.5% Triton X-100 for 5 min, and then blocked with 5% goat serum in PBS before immunostaining. Mitochondrial morphology was analyzed using an Olympus IX-70 spinning disk confocal or a Leica SP5 confocal microscope. Image deconvolution was performed using Huygens Essential software (Scientific Volume Imaging), using the batch processor with a signal to noise ratio of 8 and a maximum of 40 iterations. The output format was 16-bit, unscaled images to allow the comparison of pixel values across images (as described in Howell et al., 2009). For quantification of Flag immunofluorescence, maximum projection of all z-stacks was generated using Leica Microsystems LAS AF Software.

2.6 Drosophila apoptosis assays (performed in the laboratory of Dr. Kristen White, Harvard Medical School)

2.6.1 Wing disc apoptosis assay

Wing discs from drosophila larvae expressing dMFN under an engrailed-Gal4 driver were irradiated and stained with anti-CP32 antibody for active caspases.

2.6.2 Cell apoptosis assay

S2 cells were transfected with pMT-RPR and empty vector or pAC-dMFN. Cells were treated with increasing amounts of Cu2+ to induce Reaper expression and the percentage of apoptotic cells at various time points was measured.

2.7 Metabolic analyses

2.7.1 ATP Assays

ATP levels in siRNA-transfected Hela cells were measured in cells arrested at the G1/S boundary. Equal cells numbers were harvested following the second thymidine incubation, and relative bioluminescence was measured for triplicate samples using the ATP Bioluminescence Assay Kit CLS II (Roche).

2.7.2 Sample preparation for mass spectrometry

Hela cells that had been transfected with siRNA oligos were incubated with 1 μM L-carnitine for 20-22 h during the final thymidine block. Sterile water was added to harvested cell pellets, and cells were lysed by sonication. Protein concentrations were determined by Bradford (Biorad), and triplicate samples were prepared (with normalized protein concentrations) for analysis by mass spectrometry.

2.7.3 Mass spectrometry (performed by Sarah W. Stedman Nutrition and Metabolism Center

Acylcarnitines and amino acids were analyzed by mass spectrometry as previously described (An et al., 2004; Newgard et al., 2009).

3. Reaper-induced mitochondrial fragmentation through interaction with mitofusins Mfn2 and dMFN

3.1 Introduction

The regulated fragmentation of cellular mitochondria constitutes an important event in the apoptotic program, and this morphological change is evolutionarily conserved between worms and flies and mammals (Abdelwahid et al., 2007; Breckenridge et al., 2008; Desagher and Martinou, 2000; Frank et al., 2001; Goyal et al., 2007; Jagasia et al., 2005; Mancini et al., 1997). The dynamin-related GTPase Drp1 coordinates mitochondrial fragmentation during apoptosis, and loss of Drp1-mediated fission interferes with developmental apoptosis and normal progression of the apoptotic program. In the worm, *C. elegans,* inhibition of mitochondrial fission decreases cell death during development. The inhibition of programmed cell death during development is also observed in embryos and cells from the fly, *D. melanogaster*, when Drp1 is inactivated (either by RNAi-mediated silencing or genetic mutation) (Abdelwahid et al., 2007; Goyal et al., 2007; Jagasia et al., 2005). Cells derived from a Drp1-/- mouse display defects in the timing of cytochrome c release, as well as defective neural tube formation due to the failure of developmental apoptosis (Ishihara et al., 2009; Wakabayashi et al., 2009). Together, these data indicate an essential role for Drp1 in both caspase-initiated mitochondrial elimination as well as mitochondrial quality control via mitophagy (Breckenridge et al., 2008; Twig et al., 2008).

Mitochondrial fusion, mediated by the mitofusins Mfns1/2 and Opa 1, opposes mitochondrial fragmentation, and the balance between these processes is integral for the maintenance of cellular function. Point mutations in the fusion proteins Mfn2 and Opa1 lead to neurodegenerative diseases such as Charcot-Marie-Tooth type 2A and dominant optic atrophy, and disruption of the balance between mitochondrial fission and fusion may contribute to the pathogenesis of Parkinson's and Alzheimer's diseases (reviewed in Chen and Chan, 2009; Wang et al., 2009).

Like the fission protein Drp1, the mitochondrial fusion proteins also contribute to homeostatic changes in mitochondrial morphology as well as mitochondrial fission during apoptosis. In the absence of mitochondrial fusion (by elimination of both Mfn1 and Mfn2 or by RNAi of Opa1), cells display severe defects in growth, respiration, and mitochondrial membrane potential (Chen et al., 2005). Overexpression of the mitofusins leads to increased membrane connectivity and delays in Bax activation, cytochrome c release and cell death (Jahani-Asl et al., 2007; Neuspiel et al., 2005; Suen et al., 2008; Sugioka et al., 2004).

In contrast to these reports of a protective role for the mitofusins, Opa1 has both pro- and anti-apoptotic characteristics. Although the loss of Opa1 results in spontaneous apoptosis (an anti-apoptotic role) (Olichon et al., 2003), Opa1-mediated cristae remodeling occurs downstream of pro-apoptotic Bcl-2 proteins and is required for apoptosis (Cipolat et al., 2006; Frezza et al., 2006; Yamaguchi et al., 2008).

Initial reports describing mitochondrial fragmentation during cell death characterized colocalization of the pro-apoptotic Bcl-2 family member Bax with both the fission factor Drp1 and the fusion factor Mfn2 in fission foci during apoptosis (Karbowski et al., 2002). An understanding of the interplay between regulators of mitochondrial morphology and members of the Bcl-2 protein family is emerging, highlighting the importance of this relationship in both apoptotic mitochondrial fragmentation and in the homeostatic maintenance of dynamics (as evidenced by the defects in morphology in the Bax/Bak double knockout cells and the ability of Bcl-xL to promote both mitochondrial fission and fusion) (Berman et al., 2009; Delivani et al., 2006; Karbowski et al., 2006). Moreover, the inhibition of mitochondrial fusion at the time of Bax mitochondrial foci formation suggests that apoptotic fragmentation may reflect both the activation of fission and the inhibition of fusion prior to cytochrome c release and caspase activation (Benard and Karbowski, 2009; Brooks et al., 2007; Karbowski et al., 2004).

In contrast to the release of mitochondrial cytochrome c in vertebrates, the regulation of Drosophila inhibitor of apoptosis protein-1 (DIAP1) levels constitutes the main control point for fly apoptosis. The removal of DIAP1 from fly cells or embryos is sufficient to induce rapid apoptosis, and cytochrome c does not appear to be required for assembly of the fly apoptosome (Hay et al., 1995; Lisi et al., 2000; Muro et al., 2002; Yoo et al., 2002; Yu et al., 2006b). In Drosophila, the key inducers of apoptosis, Reaper,

Hid and Grim (RHG proteins), act by antagonizing DIAP proteins, releasing active caspases from IAP-inhibition. Interestingly, the RHG proteins localize to mitochondria, and this localization is essential for mitochondrial disruption by Reaper and Hid and for Reaper-induced DIAP1 degradation (Abdelwahid et al., 2007; Claveria et al., 2002; Freel et al., 2008; Haining et al., 1999; Olson et al., 2003).

Although mitochondrial cytochrome c release does not play a widespread role in fly cell death, the fragmentation of mitochondria during apoptosis does. In *drp1* mutant flies, mitochondria fail to fragment during normal developmental apoptosis and in response to various apoptotic stimuli (including Reaper induction) (Abdelwahid et al., 2007; Goyal et al., 2007). Failure of apoptotic mitochondrial fragmentation results in protection from cell death and tissue hyperplasia in vivo, highlighting the contribution of this evolutionarily-conserved morphological change to the execution of apoptosis.

Based on the role of Reaper in mitochondrial disruption and the suppression of Reaper-induced death in *drp1* mutant flies, we analyzed Reaper-induced changes in mitochondrial morphology. Here, we report that Reaper expression results in mitochondrial fragmentation, and we propose that this fragmentation results from Reaper-mediated inhibition of mitochondrial fusion by Mfn2 and the Drosophila Mfn2 orthologue dMFN. We show that Reaper colocalizes with and binds to the mitochondrial fusion proteins Mfn2 and dMFN. Moreover, overexpression of dMFN inhibits Reaper-induced killing both in vitro and in vivo, suggesting that active

inhibition of mitochondrial fusion by Reaper may contribute to mitochondrial fragmentation during cell death.

3.2 Results

Although mitochondrial cytochrome c release does not mediate fly apoptosis, the RHG proteins can potently trigger cytochrome c release and caspase activation both in fly cells and when expressed in vertebrate cells and cell-free lysates (Abdelwahid et al., 2007; Claveria et al., 1998; Evans et al., 1997; Goyal et al., 2007; Haining et al., 1999). This ability suggests that these proteins have the capacity to act on mitochondria. Indeed, both Reaper and Grim have a Grim Helix 3 (GH3) domain that confers mitochondrial localization and is required for full pro-apoptotic activity, and Hid contains a C-terminal domain for mitochondrial localization (Claveria et al., 2002; Haining et al., 1999; Olson et al., 2003). The GH3 domain of Reaper is also important for inducing DIAP degradation, as it confers on Reaper the ability to interact with lipids at the mitochondria, a localization necessary for efficient DIAP1 degradation (Freel et al., 2008; Olson et al., 2003).

3.2.1 Reaper induces mitochondrial fragmentation

Based on the capacity of RHG proteins to act on mitochondria and the importance of Reaper- and Hid-mediated mitochondrial disruption in the activation of fly caspases, we examined mitochondrial morphology in cells expressing Reaper. We found that Reaper induces dramatic changes in mitochondrial morphology: short,

punctuate mitochondria predominated in Hela cells expressing Reaper, whereas control cells and cells transfected with Reaper lacking its mitochondrial-targeting GH3 domain (ΔGH3, lacking amino acids 32-43) were more elongated and interconnected (Figure 3-1). These data are consistent with previous reports demonstrating that Reaper expression induces mitochondrial fragmentation (Abdelwahid et al., 2007; Goyal et al., 2007).

Upon close examination, we noticed that Reaper localizes to distinct sub-mitochondrial foci in Hela cells and in Drosophila S2 cells (Figure 3-2). Reaper foci appear to localize to mitochondrial tips and resemble the mitochondrial scission sites previously described for Bax, Drp1 and Mfn2, suggesting that Reaper (like Bax) may target to foci and participate in apoptotic scission events (Karbowski et al., 2002).

3.2.2 Reaper colocalizes with Mfn2 and dMFN

Based on this pattern of foci formation, we examined whether Reaper colocalizes with known regulators of mitochondrial dynamics, namely the fission protein Drp1 and the fusion protein Mfn2. We hypothesized that Reaper-mediated mitochondrial fission may occur through Reaper-induced activation of Drp1. However, we did not observe colocalization of Reaper with either wild-type Drp1 or the Drp1 dominant negative mutant, Drp1K38A (which elongates mitochondria through the inhibition of mitochondrial fragmentation) (Figure 3-3B and 3-3C). Although Drp1-dependent

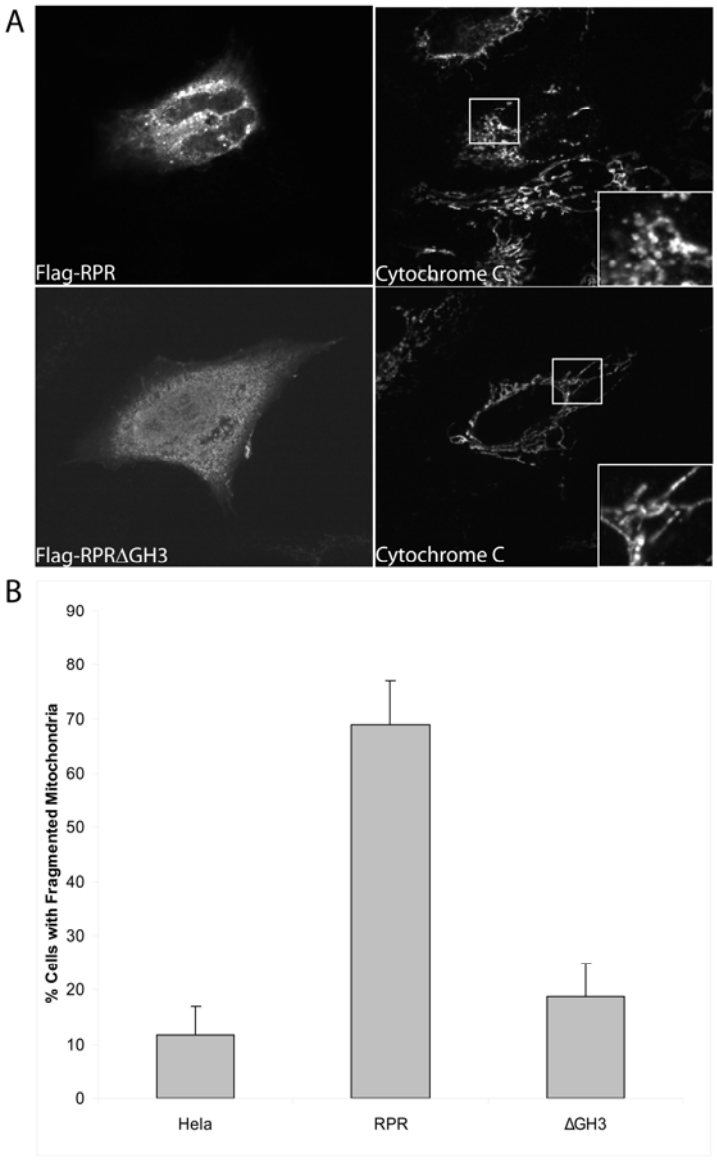


Figure 3-1: Reaper induces mitochondrial fragmentation in mammalian cells.

(A) Hela cells were transfected with pCDNA3-Flag-RPR, or pCDNA3-Flag-RPRΔGH3. Anti-Flag staining is shown on the left and anti-cytochrome c staining is shown on the right. Flag-Reaper has a punctate pattern (top) whereas ΔGH3 is more diffuse. (B) Quantification of transfected cells with fragmented mitochondria.

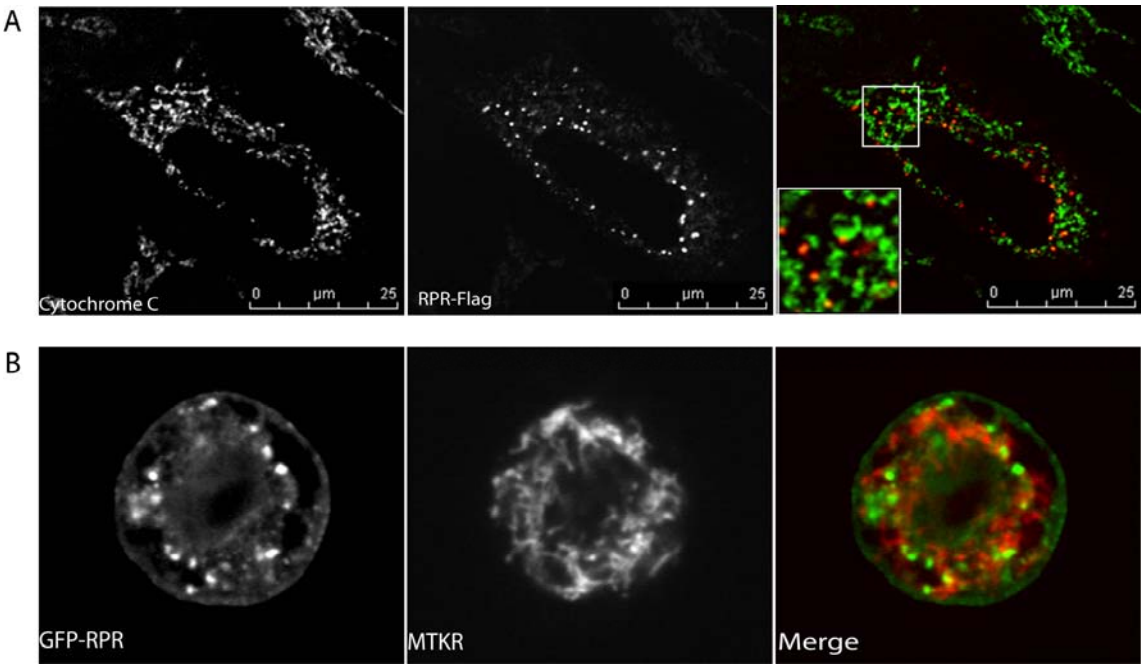


Figure 3-2: Reaper colocalizes with sub-mitochondrial fission foci.

(A) Hela cells were transfected with pEBB-RPR-Flag (red) and Mito-GFP (green). (B) S2 cells were transfected with pMT-RPR-Flag and stained with mitotracker red (MTKR).

fragmentation and apoptosis are known to occur downstream of Reaper, our data suggest that Reaper-induced fission is not likely to occur through direct activation of Drp1 by Reaper. Rather, Reaper showed striking colocalization with the fusion protein Mfn2, and the overexpression of Mfn2 appeared to enhance the localization of Reaper to mitochondria (Figure 3-3D). These observations suggest that inhibition of Mfn2-mediated mitochondrial fusion may contribute to Reaper-induced fission.

Since Reaper-induced mitochondrial alterations are important in Drosophila cell death, we wanted to examine whether Reaper colocalizes with the fly orthologue of Mfn2. The *fzo* and *dmfn* genes encode the two fly mitofusin homologue, Fzo1p and dMFN (also called Marf) (Hwa et al., 2002). Initially identified for its essential role in Drosophila sperm differentiation, the fly *fuzzy onions* gene (*fzo*) was the first mitofusin family member discovered (Hales and Fuller, 1997). Whereas expression of Fzo1p is limited to the male germline, dMFN is expressed broadly (in adult males and females as well as in S2 cells)(Hwa et al., 2002). In regards to sequence similarity, dMfn aligns most closely with Mfn2 (Figure 3-4A). dMfn also localizes to mitochondria, as shown by costaining with cytochrome c in Drosophila S2 cells (Figure 3-4B). Moreover, like Mfn2, dMFN overexpression also enhances the localization of Reaper to mitochondria, demonstrating that this aspect of Reaper function occurs in both vertebrate and invertebrate systems (Figure 3-4C).

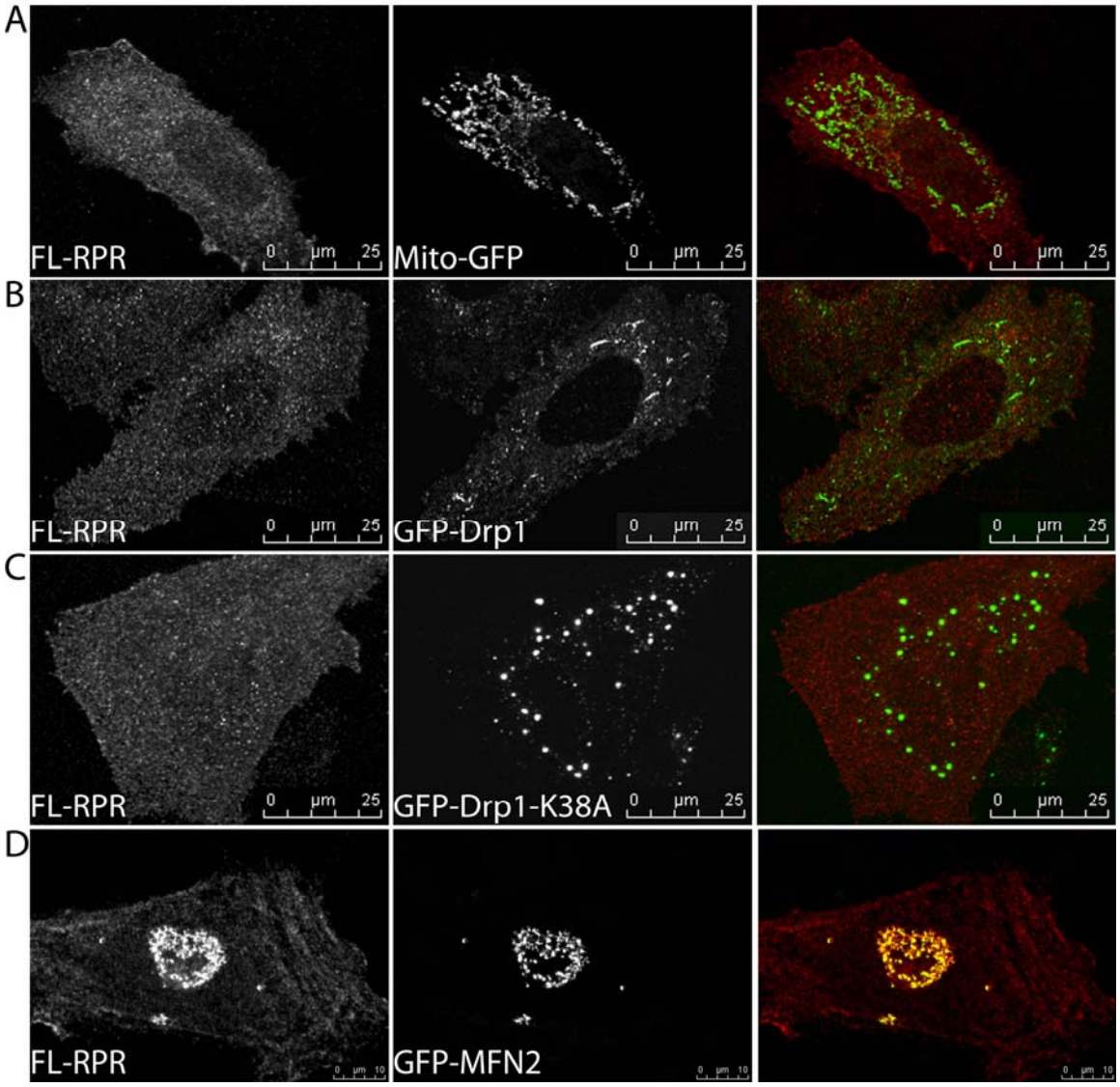


Figure 3-3: Mfn2 overexpression enhances the localization of Reaper to mitochondria.

Hela cells were co-transfected with either (A) pCDNA3-Flag-RPR and GFP (B) pCDNA3-Flag-RPR and GFP-Drp1 (C) pCDNA3-Flag-RPR and GFP-Drp1K38A or (D) pCDNA3-Flag-RPR and GFP-MFN2.

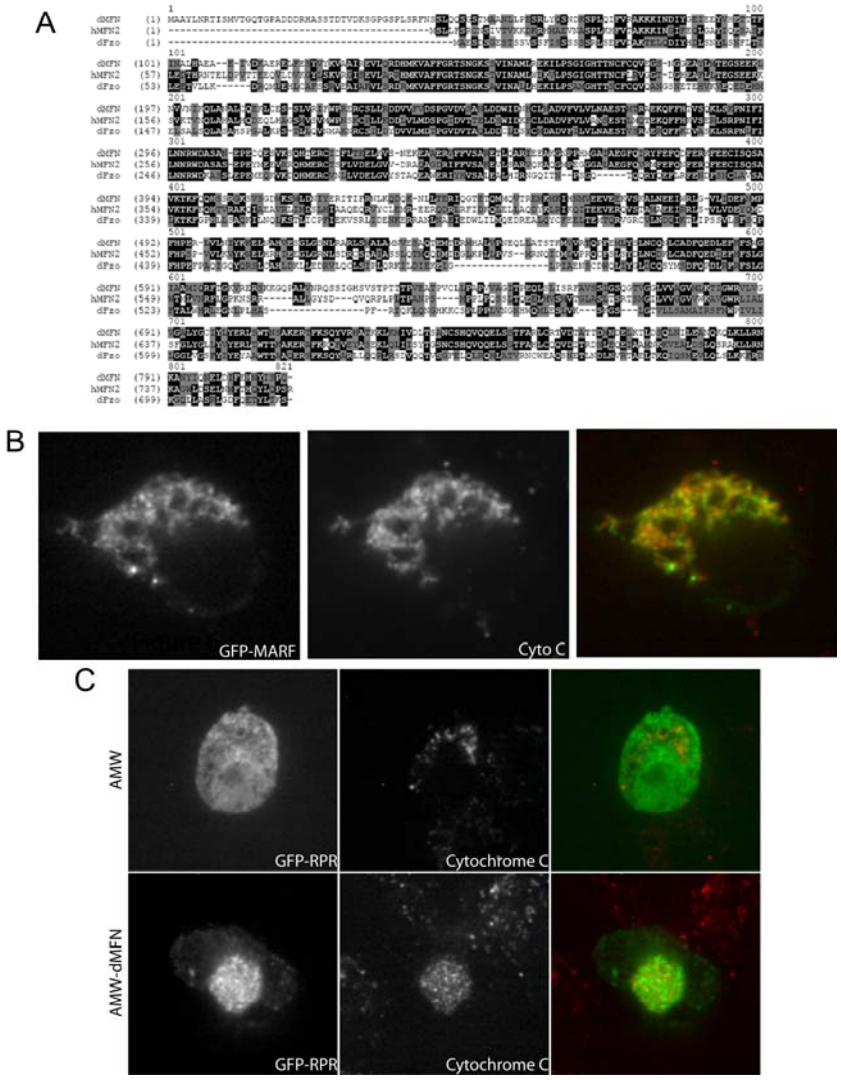


Figure 3-4: dMFN is the primary mitofusin orthologue in flies.

(A) Alignment of dMFN, hMFN2 and dFzo1. (B) S2 cells were transfected with GFP tagged dMFN and stained with cytochrome c. (C) dMFN overexpression enhances GFP-Reaper localization to mitochondria. GFP-Reaper was expressed alone (top panels) or with dMFN (bottom panels) and cells with a cytochrome c antibody for mitochondrial imaging.

We next wanted to test whether the colocalization we observed reflected a binding interaction between Reaper and Mfn2 and dMFN. To test for an interaction, we used the biochemically tractable Xenopus egg extract system as well as Drosophila S2 cell lysates. Recombinant His-tagged human Mfn2 was incubated in Xenopus egg extract treated with biotinylated reaper peptide (Figure 3-5B), and His-Mfn2 was precipitated using nickel beads. The western blot in Figure 3-5B shows that Reaper is found in the Mfn2 precipitates. Binding of endogenous Xenopus Mfn2 to a biotinylated Reaper peptide (corresponding to amino acids 16-65) was also detected. The peptide was incubated in Xenopus egg extract and precipitated with streptavidin sepharose beads, and binding of Xenopus Mfn2 to the beads by immunoblotting with an antibody raised to human Mfn2. As seen in Figure 3-5A, biotinylated Reaper peptide co-precipitated endogenous Mfn2 from Xenopus egg extract. Using this same assay, we incubated S2 cell lysates with Reaper-conjugated streptavidin beads, and we detected binding of endogenous dMFN to Reaper, indicating that this interaction also occurs in flies (Figure 3-5C).

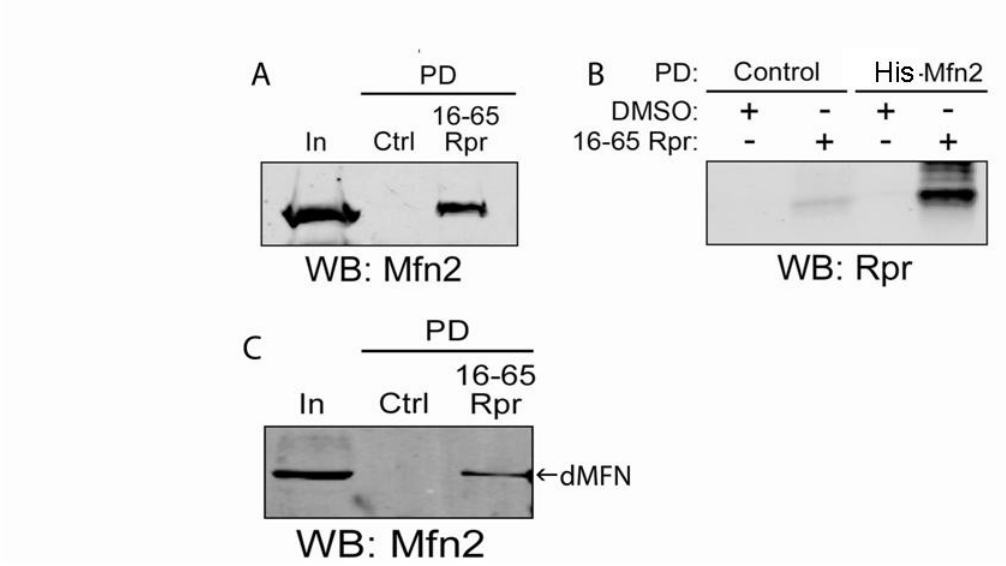
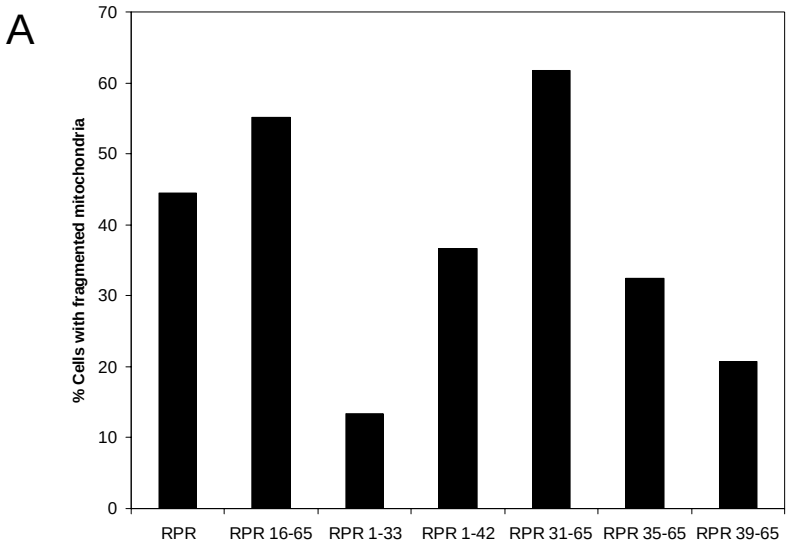


Figure 3-5: Reaper binds Mfn2 and dMFN.

(A) Biotinylated RPR-16-65 was incubated in Xenopus egg extract and precipitated with streptavidin sepharose, and precipitates were immunoblotted for Mfn2. (B) Purified His-tagged human Mfn2 was bound to nickel beads and incubated in Xenopus egg extracts treated with RPR-16-65 (or DMSO control). Immunoblotting for Reaper is shown.(C) Streptavidin sepharose-conjugated biotinylated RPR-16-65 was incubated in Drosophila S2 cell lysates and and precipitates were immunoblotted for dMFN.

3.2.3 Reaper GH3 domain is required for colocalization with Mfn2 and Reaper-induced mitochondrial fragmentation

To further dissect the domains in Reaper that confer binding to Mfn2/dMFN, we constructed a series of Reaper mutants and screened them for colocalization with Mfn2 in Hela cells. We suspected that colocalization of Reaper with Mfn2 would be independent of its N-terminal IAP-binding motif (IBM), as the report by Dr. Kristen White and colleagues had previously demonstrated that the IBM is dispensable for Reaper-mediated mitochondrial disruption by Reaper (Abdelwahid et al., 2007). Indeed, we found that a Reaper mutant lacking its IBM (Reaper 16-65) was not impaired in its ability to induce mitochondrial fragmentation; it actually resulted in a slightly higher percentage of cells with fragmented mitochondria than wild-type Reaper (55% vs. 44%, graph in Figure 3-6A). We narrowed down the region required for colocalization with Mfn2 to the GH3 domain of Reaper, the region previously shown to be important for its mitochondrial localization and for its ability to induce mitochondrial disruption and caspase activation (Abdelwahid et al., 2007; Freel et al., 2008; Olson et al., 2003). Even small truncations within this domain significantly attenuated both the colocalization of reaper with Mfn2 and its ability to induce mitochondrial fragmentation (Figure 3-6); these data suggest that mitochondrial localization of Reaper is required for its ability to both colocalize with Mfn2 and to induce mitochondrial fragmentation.



B

GH3

Colocalization

1-mavafyipdqatllreaeqkeqqilrlresqwrflatvvletlrqytschpktgrksgkyrkpsq-65 +
16-eaeqkeqqilrlresqwrflatvvletlrqytschpktgrksgkyrkpsq-65 +
1-mavafyipdqatllreaeqkeqqilrlresqwr-33 -
1-mavafyipdqatllreaeqkeqqilrlresqwrflatvvlet-42 +
31-qwrflatvvletlrqytschpktgrksgkyrkpsq-65 +
35-latvvletlrqytschpktgrksgkyrkpsq-65 -
39-vletlrqytschpktgrksgkyrkpsq-65 -

Figure 3-6: GH3-mediated mitochondrial targeting of Reaper is essential for colocalization with Mfn2 and mitochondrial fragmentation.

(A) Hela cells were transfected with the indicated Reaper mutants, and the percentage of cells with fragmented mitochondria was measured. (B) Schematic of the Reaper mutants analyzed, with the GH3 domain shown in red. Colocalization with Mfn2 is also indicated by (+) or (-).

3.2.4 dMFN inhibits Reaper-induced death in the fly

In flies, mutations in *drp1* confer protection from a range of apoptotic insults, including normal developmental cues, irradiation and etoposide treatment (Abdelwahid et al., 2007; Goyal et al., 2007). We similarly wanted to test whether dMFN overexpression attenuated Reaper-dependent apoptosis. Shown in Figure 3-7A, wing discs from Drosophila larvae expressing dMFN under an engrailed-Gal4 driver were irradiated and stained with anti-CP32 antibody for active caspases. Wing discs expressing dMFN showed a marked reduction in the number of apoptotic cells in the UAS-dMFN wing disc as compared to control larvae. In S2 cells, overexpression of dMFN also suppressed Reaper-induced apoptosis. Specifically, cells were transfected with pMT-RPR and either pAC-dMFN or vector control, and cells were treated with increasing amounts of Cu2+ to induce Reaper expression. Cell death was measured, and the percentage of apoptotic cells per condition is shown graphically (Figure 3-7B). Together, these data show that dMFN expression suppresses Reaper-induced apoptosis both in wing discs and in S2 cells, consistent with previous reports demonstrating the importance of apoptotic mitochondrial scission in Drosophila apoptosis. As in vertebrate systems, overexpression of the fly mitofusin, dMFN, can inhibit apoptosis in the fly.

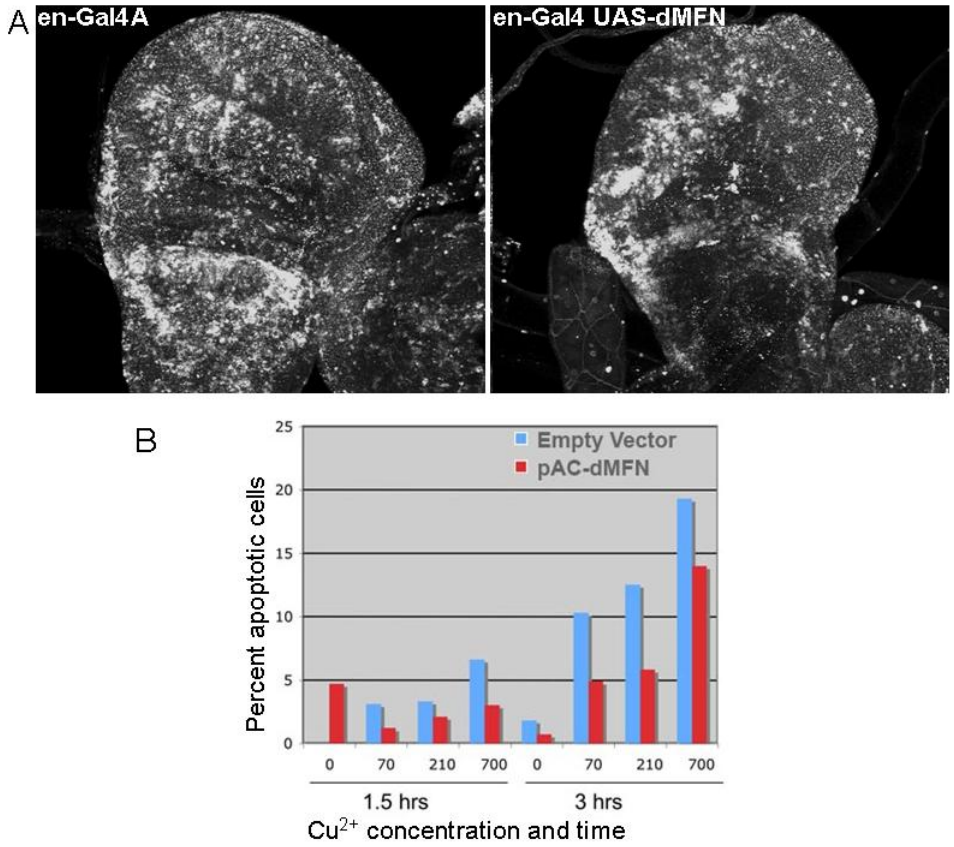


Figure 3-7: dMFN suppresses Reaper-induced apoptosis in the fly.

(A) Wing discs from drosophila larvae expressing dMFN under an engrailed-Gal4 driver were irradiated and stained with anti-CP32 antibody for active caspases. Fewer apoptotic cells are seen in the UAS-dMFN flies. (B) S2 cells were transfected with pMT-RPR and empty vector or pAC-dMFN. Cells were treated with increasing amounts of Cu^{2+} to induce Reaper expression and the percentage of apoptotic cells was measured.

3.3 Discussion

The discovery that mitochondrial fragmentation occurs closely with cytochrome c release revealed a connection between the regulation of morphology and well characterized events of apoptosis (reviewed in Benard and Karbowski, 2009; Youle and Karbowski, 2005). Previous works and our findings suggest that apoptotic scission involves both the activation of mitochondrial fission (as through Bax/Bak's promotion of Drp1's association with the mitochondrial membrane) as well as the inhibition of Mfn1/2-dependent mitochondrial fusion (Benard and Karbowski, 2009; Brooks et al., 2007; Wasiak et al., 2007). Multiple events are required for Mfn-dependent fusion to occur, including the formation of homo- or heterotypic Mfn complexes between adjacent organelles as well as GTP binding and hydrolysis. Additionally, a number of other proteins have been shown to either interact with or function downstream of the mitofusin proteins, (including the Bcl2-family members Bcl2, BclxL, Bax and Bak) as well as MIB (Mfn binding protein), Stoml2/SLP2, and mitoPLD, and several of these protein interactions influence mitochondrial morphology in an Mfn2-dependent manner (Brooks et al., 2007; Choi et al., 2006; Delivani et al., 2006; Eura et al., 2006; Hajek et al., 2007; Suen et al., 2008; Tondera et al., 2009).

Based on these known parameters of Mfn2 regulation and activity, we are currently investigating the mechanisms by which Mfn2/dMFN-dependent fusion activities may be inhibited during Reaper-induced mitochondrial fragmentation.

Though our preliminary observations do not indicate that Reaper affects the formation of Mfn2 homo-oligomers or the efficiency of GTP binding by Mfn2, we have not excluded the possibility that Reaper functions at a different step during the fusion process (such as through the recruitment of inhibitory cofactors) to suppress mitochondrial fusion.

Post-translational modifications are also important in regulation of the fission/fusion machinery. The yeast mitofusin Fzo1 is regulated by ubiquitylation and degradation, and treatment with proteasome inhibitors leads to increases in protein levels of Mfn1 and Mfn 2, suggesting that this pathway also regulates the mammalian orthologues (Neutzner et al., 2008). Based on Reaper's known ability to stimulate the ubiquitin ligase activity of DIAP1 (Ryoo et al., 2002), it is possible that Reaper works in conjunction with a Mfn2/dMFN-directed ubiquitin ligase (such as MARCH V or Parkin) to stimulate the turnover of the fusion protein during apoptosis (Nakamura et al., 2006; Yonashiro et al., 2006; Park et al., 2010; Ziviani et al., 2010).

We observed that mitochondrial fragmentation induced by Reaper is independent of its ability to inhibit DIAP1, as Reaper lacking its IBM induced mitochondrial fission as efficiently as wild-type Reaper (Figure 3-6). This finding is consistent with the initial report of Reaper-induced mitochondrial disruption, which also characterized Reaper-induced mitochondrial fragmentation and permeablization as an additional, pro-apoptotic function of this multi-faceted protein (Abdelwahid et al.,

2007). Though the role of apoptotic mitochondrial permeabilization in fly apoptosis is still unclear, it is possible that Reaper- or Hid-mediated permeabilization releases additional factors for propagation of the apoptotic signal (such as the DIAP1 antagonist, dOmi) (Challa et al., 2007; Khan et al., 2008). It is also possible that mitochondrial disruption by Reaper and Hid marks an irreversible step in the cell death program; by inhibiting normal mitochondrial function, cellular viability becomes impossible.

A requirement for mitochondrial fragmentation during apoptosis has been verified in a variety of experimental systems; inhibiting Drp1 function (through genetic deletion or mutation, or via the use of a dominant- negative Drp1 construct, a chemical inhibitor of Drp1 or through RNAi-mediated silencing of Drp1) decreases cellular apoptosis (Abdelwahid et al., 2007; Cassidy-Stone et al., 2008; Frank et al., 2001; Goyal et al., 2007; Jagasia et al., 2005; Lee et al., 2004; Wakabayashi et al., 2009). Despite the unclear role for mitochondria in fly cell death, two different laboratory groups discovered that mitochondrial fission occurs in response to genotoxic stresses and is required for apoptosis in the fly, though the authors of these reports offer different conclusions as to the role of caspase activity in mitochondrial fragmentation during apoptosis (Abdelwahid et al., 2007; Goyal et al., 2007). Abdelwahid et al. conclude that caspases contribute to mitochondrial disruption by Reaper and Hid, whereas Goyal et al. observe mitochondrial fragmentation upstream of effector caspase activation. Using *C. elegans*, Breckenridge et al. identified a novel pro-apoptotic role for Drp1 that is

actually downstream of caspase activation (Breckenridge et al., 2008; Jagasia et al., 2005). These authors propose that the primordial role for Drp1-dependent fission in apoptosis might be to eliminate mitochondria following caspase activation, and any role for Drp1 in mediating cytochrome c release in mammals might have been acquired during evolution. In this evolutionary context, it is interesting to consider that Reaper-mediated inhibition of mitochondrial fusion might play a role in mitochondrial inactivation during cellular disassembly.

A role is emerging for the mitochondrial fission/fusion machinery in the selective elimination of dysfunctional mitochondria through autophagy (also called mitophagy). Depolarized mitochondria are selectively excluded from mitochondrial fusion events and are targeted for autophagy via mitochondrial fission, and genetic data in the fly support an interaction between the genes required for mitochondrial fission and fusion and the Parkin-mediated mitophagy pathway (Deng et al., 2008; Narendra et al., 2008; Poole et al., 2008; Twig et al., 2008; Ziviani et al.). Both during apoptosis and prior to mitochondrial elimination in mitophagy, mitochondrial fusion is blocked. Thus, the possibility exists that Reaper-mediated inhibition of fusion is involved in mitochondrial elimination via mitophagy, and future experiments will be aimed at determining whether Reaper is involved in mitochondrial clearance.

Our lab and others have shown that the mitochondrial localization of the RHG proteins is essential for their maximal apoptotic potential (Abdelwahid et al., 2007;

Claveria et al., 2002; Freel et al., 2008; Haining et al., 1999; Olson et al., 2003). For Reaper, the GH3 domain confers lipid interactions required for both membrane localization and DIAP1 degradation (Freel et al., 2008; Olson et al., 2003). Here, we define another function of the GH3 domain in Reaper; it confers colocalization with the mitofusins Mfn2 and dMFN, resulting in GH3-dependent mitochondrial fission (likely due to inhibition of mitochondrial fusion). dMFN inhibits Reaper-induced cell death in the fly and in fly S2 cells, further demonstrating the importance mitochondrial fission to the cell death program in Drosophila. Together with previous reports of Reaper-induced mitochondrial disruption, our data suggest that Reaper-mediated inhibition of fusion (and the resulting mitochondrial fission) may serve to both propagate the apoptotic signal (through the release of pro-apoptotic factors) and to facilitate the elimination of mitochondria. The regulation of mitochondrial dynamics by pro-apoptotic signals like the RHG proteins supports a more general role for regulated mitochondrial fission during apoptosis that is conserved throughout evolution.

4. Regulation of mitochondrial morphology and metabolism by APC/C^{Cdh1}-mediated control of Drp1 stability

This chapter is adapted from a manuscript currently in revision.

4.1 Introduction

Homeostatic maintenance of cellular mitochondria requires a dynamic balance between fission and fusion, and controlled changes in morphology are important for processes like apoptosis and cellular division. Interphase mitochondria have been described as an interconnected network that fragments as cells enter mitosis, and this mitotic mitochondrial fragmentation is known to be regulated by phosphorylation and deSUMOylation of the dynamin-related GTPase Drp1, a key component of the mitochondrial division machinery (Hoppins et al., 2007; McBride et al., 2006; Okamoto and Shaw, 2005; Taguchi et al., 2007; Zunino et al., 2009). Loss of Drp1 function and the subsequent failure of mitochondrial division during mitosis leads to incomplete cytokinesis and the unequal distribution of mitochondria into daughter cells (Ishihara et al., 2009; Labrousse et al., 1999; Okamoto and Shaw, 2005; Taguchi et al., 2007). During mitotic exit and interphase, the mitochondrial network reforms. Significant increases in mitochondrial membrane potential and respiration occur during progression through G1, and the presence of a hyperfused mitochondrial network is necessary for cyclin E accumulation and cell cycle progression (Mitra et al., 2009; Schieke et al., 2008). Here, we

demonstrate that changes in mitochondrial dynamics as cells exit mitosis are driven in part through ubiquitylation of Drp1, catalyzed by the APC/C^{Cdh1} (anaphase-promoting complex/cyclosome and its coactivator Cdh1) E3 ubiquitin ligase complex during mitotic exit. Importantly, inhibition of Cdh1-mediated Drp1 ubiquitylation and proteasomal degradation during interphase prevents the normal regrowth of mitochondrial networks following cell division.

4.2 Results

4.2.1 Drp1 levels decrease upon release from mitotic arrest

Mitochondrial fragmentation at the time of mitotic entry is known to be regulated by Cdk1/cyclin B-mediated phosphorylation and SenP5-mediated deSUMOylation of Drp1 (Taguchi et al., 2007; Zunino et al., 2009). Given the dramatic shift in morphology from fragmented mitochondria in mitosis to a predominantly elongated mitochondrial network during interphase, we wanted to determine whether alterations in Drp1 also contributed to changes in mitochondrial morphology as cells exited mitosis and entered G1. Based on the importance of ubiquitin-dependent degradation in coordinating key cell-cycle transitions, we questioned whether the dramatic changes in mitochondrial morphology that occur as cells undergo division are regulated by proteolytic degradation of Drp1. We examined the stability of Drp1 during cell cycle progression and found that Drp1 levels dropped when cells were released from a nocodazole-induced pro-metaphase arrest, as the cells exited mitosis and entered

G1 (Figure 4-1A). Additionally, levels of Drp1 gradually increased as Hela cells entered mitosis following release from a double thymidine block (G1/S arrest), whereas levels of several other proteins involved in the regulation of mitochondrial morphology did not change (Figure 4-1B). We noted that this downregulation of Drp1 temporally coincides with the return of mitochondria to a reticular network from their predominantly fragmented mitotic morphology (Figure 4-2). Additionally, we found that in vitro translated ^{35}S Drp1 was degraded in cell extracts prepared from synchronized G1 cells, whereas Drp1 was stable in lysates prepared from prometaphase-arrested cells (Figures 4-1C and 4-1D).

In an in vitro mitotic exit assay, Drp1 protein levels decreased, whereas blocking mitotic exit with addition of the APC/C inhibitory protein Emi2 prevented Drp1 degradation (Figure 4-3A). Based on the timing of Drp1 degradation and the ability of Emi2 to interfere with this degradation, we examined the possibility that Drp1 might be ubiquitylated by the APC/C ubiquitin ligase, an E3 complex active during mitotic exit and the G1 phase of the cell cycle. In this regard, we were interested to find that Drp1 contains multiple APC/C degradation motifs, including a canonical D-box motif (R-X-X-L-X-X-X-X-N/D/E), suggesting that it might be a substrate of the APC/C (Figure 4-3B).

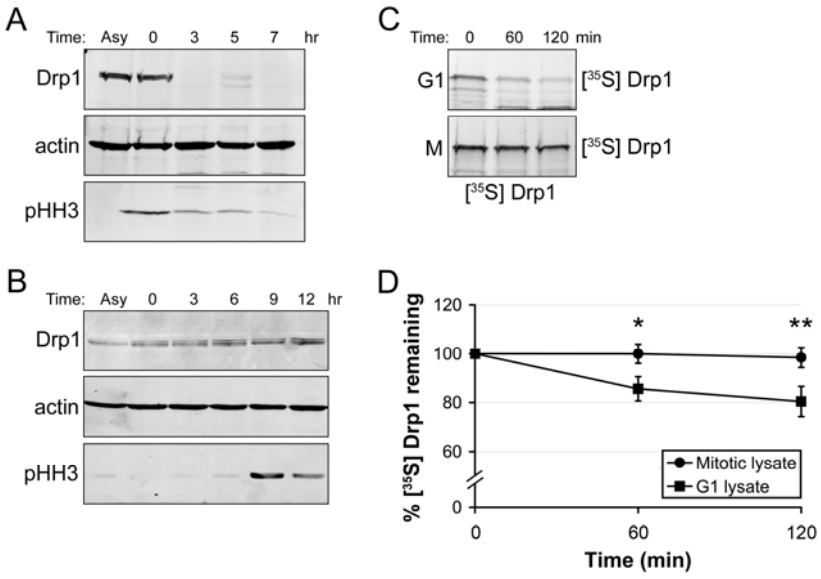


Figure 4-1: Drp1 levels decrease upon release from mitotic arrest.

Representative gels and western blots showing: (A) prometaphase-arrested Hela cells were collected at the indicated times after nocodazole release. Actin is shown as a loading control, and phospho-Histone H3 is a mitotic marker; (B) Hela S3 cells were released from thymidine-induced G1/S arrest into media containing nocodazole and were collected at the indicated times; and (C) Autoradiograms of ^{35}S-labelled Drp1 after incubation with Hela cell extracts prepared two hours after release from nocodazole arrest, or from Hela cells arrested in prometaphase with nocodazole. (D) Quantification of data shown in (C), n=5. Differences in the percentage of ^{35}S-Drp1 remaining in G1 lysates versus the mitotic lysates at timepoints 60 and 120 min are statistically significant by Student's *t* test (*, p=0.013079, and **, p=0.013553).

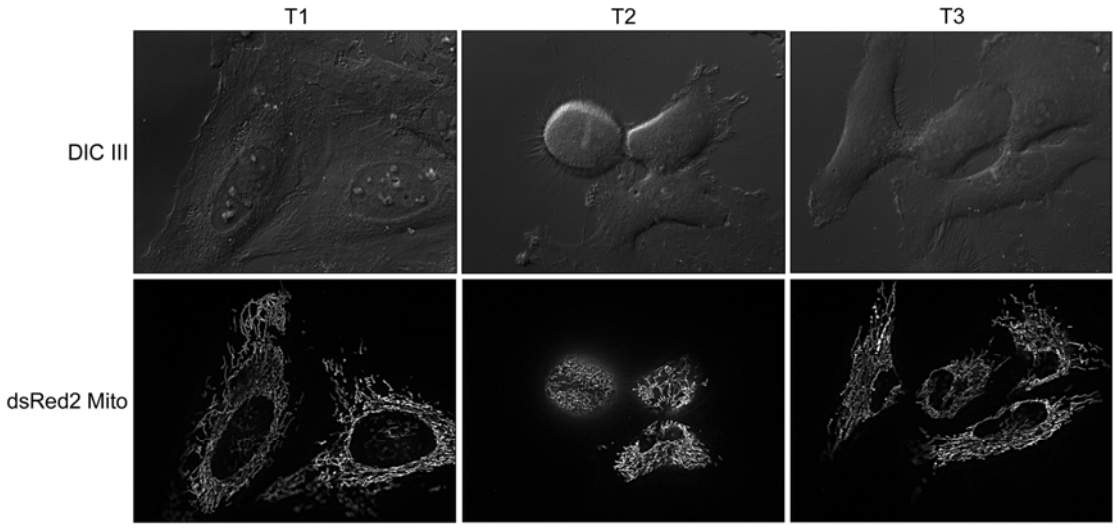


Figure 4-2: Distinct mitochondrial morphologies during interphase and mitosis.

DIC III imaging and dsRed2 mitochondrial staining of Hela cells released from arrest at the G1/S boundary with a double thymidine block. Cells in the far left panels (T1) are in G2, cells the center panels (T2) are entering mitosis, and cells in the right panels are in G1 (T3).

4.2.2 Drp1 is a novel substrate of the APC/C

Drp1 levels are lowest during the G1 phase of the cell cycle, when the APC/C is active through its association with Cdh1. We examined the effect of over-expression of the APC/C co-activator proteins Cdc20 and Cdh1 on Drp1 levels and found that Cdh1 expression resulted in a significant decrease in steady-state levels of Drp1 (Figure 4-3C). Importantly, this decrease in Drp1 was attenuated by treatment with the proteasome inhibitor MG132. We also detected binding between Drp1 and Cdh1 in cell lysates (Figures 4-3D and 4-3E). Based on the role of Drp1 in mediating fission of mitochondria, we examined mitochondrial morphology in cells over-expressing Cdh1 and found that over-expression of Cdh1 results in a range of alterations in mitochondrial morphology that are all consistent with decreased mitochondrial fission (Figure 4-3F).

4.2.3 Drp1 D-Box contributes to APC/C^{Cdh1}-mediated ubiquitylation and degradation

To examine the contribution of the putative Drp1 D-Box to Drp1 degradation by APC/C^{Cdh1}, we performed in vitro ubiquitylation assays using wild-type Drp1 or a Drp1 in which two key D-Box residues had been mutated to alanines (RxxL to AxxA). When supplemented with recombinant Cdh1, APC complexes immunoprecipitated from interphase extracts prepared from Xenopus eggs (a rich source of APC components) promoted polyubiquitylation of wild-type Drp1, whereas ubiquitylation of Drp1 containing a mutant D-box motif was attenuated, though not completely abolished (Figure 4-4B; see more below).

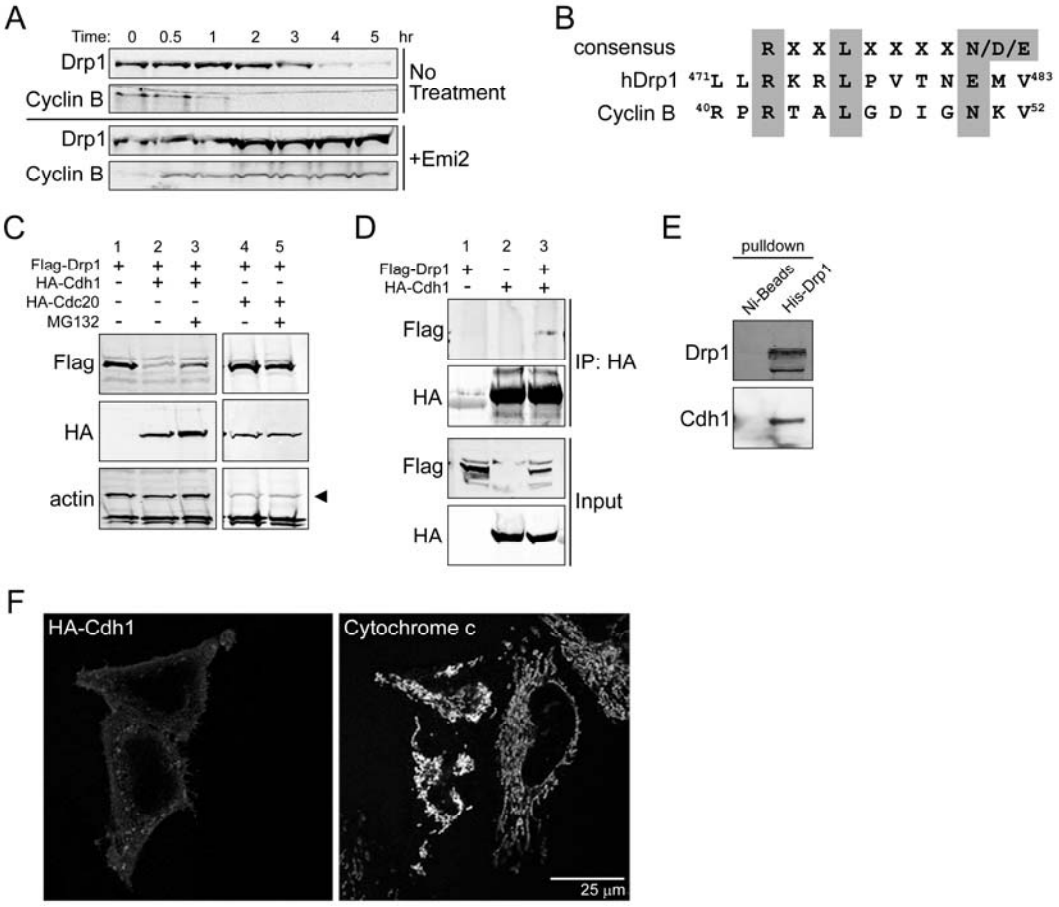


Figure 4-3: Drp1 degradation is stimulated by APC/C^{Cdh1}.

(A) HelaS3 cells were arrested with nocodazole and then cultured in fresh media for 1 hour. Hypotonic cell lysates were incubated at room temperature and aliquots were taken at the indicated times for immunoblotting. In the bottom panel (+ Emi2), GST-Emi2 was added to cell lysates before incubation at room temperature. (B) Alignment of D-boxes in Drp1 and Cyclin B1. (C) Expression of Flag-tagged Drp1 with or without HA-Cdh1 or HA-Cdc20 in 293T cells, in the absence or presence of the proteasome inhibitor MG132. (D) HA-Cdh1 and Flag-Drp1 were expressed in 293T cell extracts, and the proteasome inhibitor epoxomicin was added 11 h before cells were collected. Cell lysates were immunoprecipitated (IP) with an antibody against HA and analyzed by immunoblotting. (E) Ni-beads or Ni-beads conjugated to His-tagged Drp1 were incubated with Hela cell lysates, and the beads were immunoblotted for Cdh1. (F) Cytochrome c staining of mitochondrial morphology in asynchronous Hela cells transfected with HA-Cdh1 (stained for HA, shown in green) or untransfected (no HA staining). Images shown are representative of multiple independent experiments.

We next compared the expression of Drp1 WT to the Drp1 D-Box mutant during G1 phase and found that the amount of D-Box mutant protein remaining after release from nocodazole arrest was increased relative to the wild-type protein (Figure 4-4C). Although levels of the D-Box mutant were stabilized during G1 relative to the wild-type protein, partial degradation of the D-Box mutant still occurred, suggesting that additional APC targeting motifs may be required for full degradation, as is the case for other for APC/C^{Cdh1} substrates such as Claspin and Aurora A (Bassermann et al., 2008; Crane et al., 2004).

Upon close examination of the Drp1 protein sequence, we noted that it contains several previously described degrons for APC/C substrates, including nine canonical RxxL motifs as well as non-canonical motifs such as a GxEN box and an O-Box (Araki et al., 2005; Castro et al., 2003), and it is likely that multiple degrons are necessary for full Drp1 degradation by APC/C^{Cdh1}. We analyzed a series of Drp1 mutants to determine whether additional residues contribute degradation by APC/C^{Cdh1}-mediated degradation, and these experiments confirmed that additional residues are likely necessary for full Drp1 degradation (Figure 4-5). Still, among the additional RxxL point mutants tested, the single D-Box mutant consistently showed the largest attenuation of degradation in the presence of Cdh1, confirming that this degron is necessary for degradation by the APC/C.

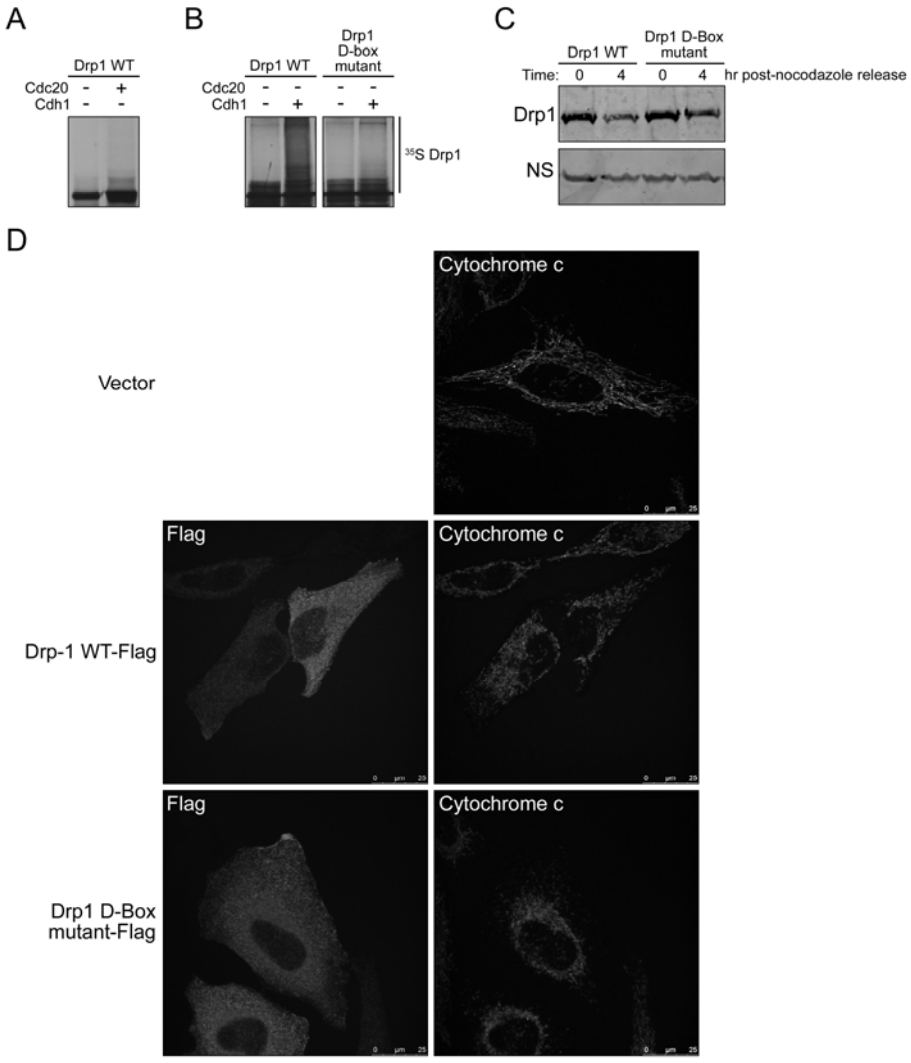
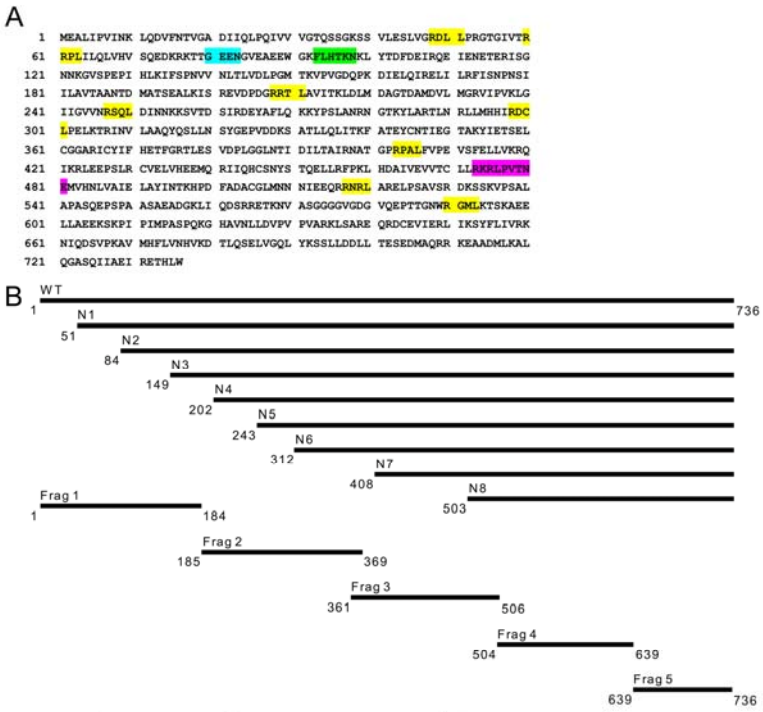


Figure 4-4: Requirements for APC/C^{Cdh1}-mediated degradation of Drp1.

(A) Ubiquitylation of in vitro translated ^{35}S Drp1 (wild-type or D-box mutant) by immunopurified APC/C supplemented with recombinant Cdc20 (A) or Cdh1 (B). (C) Hela lysates expressing either Drp1 wild-type or Drp1 D-box mutant at the indicated times after release from nocodazole arrest. A non-specific band (NS) of approximately 30 kD is shown as a loading reference. (D) Flag and Cytochrome c staining of mitochondrial morphology in a subset of early G1-phase Hela cells transfected with either Flag-tagged pcDNA3-Drp1WT or pcDNA3-Drp1 D-box mutant (versus untransfected cells, showing no Flag staining in top panel.



Construct:	Expression in HEK 293T cells:	Degradation by Cdh1:
WT	+	+
N1	+	+
N2	- (Not observed)	
N3	+	+
N4	- (Not observed)	
N5	- (Not observed)	
N6	+	+
N7	+	+
N8	+	+
Fragment 1	+	+
Fragment 2	- (Not observed)	
Fragment 3	+ (Low)	-
Fragment 4	+	+

Figure 4-5: Additional degrons are necessary for full Drp1 degradation.

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(A) Amino acid sequence alignment of human, mouse, Xenopus and yeast Drp1/Dnm1p proteins. Potential APC/C degrons are highlighted as follows: minimal D-box motifs (RxxL) in yellow, GxEN box motif in blue, the O-Box motif in green, and the canonical D-Box (RxxLxxxxN/D/E) in pink. The amino acid sequences were aligned with the AlignX program in Vector NTI (Invitrogen). (B) Schematic of Drp1 mutants constructed and analyzed for Cdh1-mediated degradation. (C) Summary of Drp1 mutants analyzed for degradation by HA-Cdh1 when coexpressed in 293T cells.

Since the overexpression of wild-type Drp1 alone is sufficient to induce mitochondrial fragmentation, we anticipated that it would be difficult to distinguish differences in degrees of mitochondrial fragmentation in cells overexpressing the wild-type Drp1 versus the Drp1 D-Box mutant (Jagasia et al., 2005; Labrousse et al., 1999; Szabadkai et al., 2004). Indeed, when we examined cells in early G1 phase that had been transfected with low levels of plasmid encoding either a Drp1 wild-type FLAG-tagged construct or a Drp1 D-Box mutant FLAG-tagged construct, we observed significant mitochondrial fragmentation with expression of both the wild-type and D-Box mutant constructs. When mitochondrial morphology was examined in early G1 cells (when Cdh1 activity is highest), the expression of either construct (at approximately equal levels, based on quantification of FLAG fluorescence) resulted in a similar degree of mitochondrial fragmentation (Figure 4-4D). Together, these data demonstrate that, like the wild-type protein, the D-Box mutant protein is functional in inducing mitochondrial fragmentation (in contrast to other known mutations within this domain of Drp1, which have a dominant negative effect and actually block mitochondrial fission) (Waterham et al., 2007).

We do consistently see increased expression of the D-Box mutant relative to the wild-type in overexpression experiments, and we see decreased ubiquitylation of the D-Box mutant, all consistent with an overall increase of the D-Box mutant protein relative to the wild-type. However, we believe that expression of the wild-type protein (at levels

comparable to the D-Box mutant protein) results in a comparable degree of mitochondrial fission. For this reason, we wanted to investigate the effects of Cdh1-mediated Drp1 degradation in a Cdh1 loss-of-function context, as a cleaner way of addressing the physiological relevance of this degradation event.

4.2.4 Cdh1 regulates Drp1-dependent changes in mitochondrial morphology and metabolism during interphase

Consistent with a role for APC/C^{Cdh1} in the degradation of Drp1, we detected elevated Drp1 protein levels in mouse embryonic fibroblasts (MEFs) generated from a Cdh1-null mouse, as has been reported for other Cdh1 substrates (Figure 4-6A) (Garcia-Higuera et al., 2008; Li et al., 2008). Moreover, when we immunoprecipitated Drp1 from either Cdh1 +/+ or -/- MEF cell lysates, we noted a decrease in Drp1 ubiquitylation in the absence of Cdh1 (although some ubiquitylation still occurred, consistent with previous reports of other E3 ubiquitin ligases targeting Drp1 (Karbowski et al., 2007; Nakamura et al., 2006; Yonashiro et al., 2006) (Figure 4-6B). We next compared levels of Drp1 protein during G1 phase in cell lysates generated from either the Cdh1 +/+ MEFS versus the Cdh1 -/- MEFs, and we found that the amount Drp1 protein remaining in the Cdh1 -/- cell lysates after release from nocodazole arrest was increased relative to the wild-type Cdh1 +/+ MEF lysates (Figure 4-6C). Together, these data demonstrate that Drp1 ubiquitylation and degradation is decreased in the absence of Cdh1.

Based on our observation that over-expression of Cdh1 decreases Drp1 protein levels and promotes a more interconnected mitochondrial network, we wished to

investigate the effects on mitochondrial morphology and function of an acute knockdown of Cdh1 in synchronized cells. Accordingly, we silenced Cdh1 expression (Figure 4-6D), and then we measured mitochondrial morphology in a population of Hela cells arrested at the G1/S border. During this point in the cell cycle, mitochondria normally form an elongated network, as we saw in our scrambled control RNAi. RNAi-mediated silencing of Cdh1 resulted in the predominance of short, punctate mitochondria (Figure 4-6E, upper panels). This mitochondrial fragmentation induced by Cdh1 RNAi was dependent on Drp1, as knocking down both Cdh1 and Drp1 attenuated this effect (Figure 4-6E, lower panels).

4.3 Discussion

Based on the above results, APC/C^{Cdh1}-mediated degradation of Drp1 appeared to underlie the morphological changes occurring in the mitochondria following cell division. It has been suggested that the transition from an interconnected mitochondrial network during interphase to a fragmented morphology during mitosis might not only function to physically separate mitochondria into daughter cells, but might also reflect variations in energy needs and utilization during the cell cycle (Benard and Rossignol, 2008; Grandemange et al., 2009). The recent identification of retrograde signaling pathways from the mitochondria has uncovered a metabolic checkpoint at the G1/S boundary, highlighting the importance of ensuring that cellular proliferation is

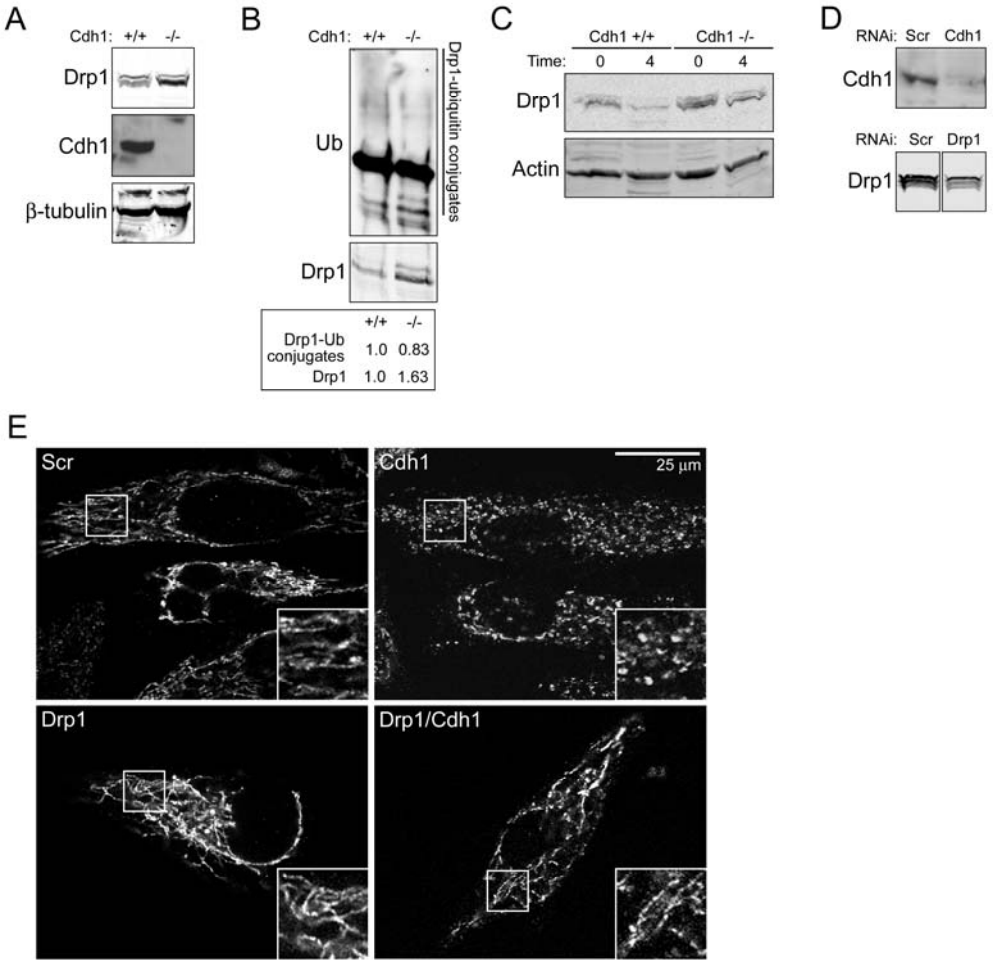

Figure 4-6: Cdh1 regulates Drp1-dependent changes in mitochondrial morphology during interphase.

(A) Immunodetection of Cdh1 and Drp1 in lysates prepared from proliferating wild-type or Cdh1-null MEFs. β-tubulin is shown as a loading control. (B) Drp1 immunoprecipitates generated from wild-type or Cdh1-null MEFs immunoblotted for ubiquitin. (C) Lysates prepared from either wild-type or Cdh1-null MEFS at the indicated times after release from nocodazole arrest were immunoblotted for Drp1. (D) Hela cells were tranfected with siRNA corresponding to a non-relevant mRNA (Scrambled, Scr) or siRNAs targeting Cdh1 or Drp1 mRNA, and lysates were prepared from G1/S-arrested cells for immunoblotting. (E) Cytochrome c staining of mitochondrial morphology in G1/S-arrested cells transfected with the following siRNA oligonucleotides: Scrambled, Cdh1, Drp1, and both Drp1 and Cdh1.

coordinated with sufficient metabolic capacity and proper mitochondrial function (Jones et al., 2005; Mandal et al.; Mandal et al., 2005; Mitra et al., 2009; Owusu-Ansah et al., 2008).

Although the relationships between morphology and mitochondrial metabolism are not fully understood, the decrease in levels of Drp1 protein temporally coincided with the return of mitochondria to a reticular morphology and with the significant increases in membrane potential and respiration that occur during progression through G1 (Schieke et al., 2008). A hyperfused mitochondrial state at G1/S is necessary for cyclin E accumulation and cell cycle progression, and Drp1-dependent mitochondrial fragmentation induced by the novel MTGM protein halts cells in the S phase of the cell cycle (Mitra et al., 2009; Zhao et al., 2009). Thus, we hypothesized that APC/C^{Cdh1}-mediated degradation of Drp1 (and the resulting re-establishment of the mitochondrial network) might function to serve the metabolic demands of a proliferating cell.

Based on the recent report that a hyperfused mitochondrial state at G1/S regulates cyclin E accumulation, we tested whether the acute knockdown of Cdh1 (and the resulting mitochondrial fragmentation) would consequently decrease levels of cyclin E. Indeed, we saw a decrease in cyclin E levels in Cdh1-RNAi cells relative to RNAi control cells both in early G1 cells (Figure 4-7B) and in cells arrested at G1/S (data not shown). Importantly, this decrease was attenuated in the presence of the double

Cdh1/Drp1 knockdown, suggesting that the changes in mitochondrial morphology correlate with cyclin E levels.

We next wanted to determine whether cellular ATP levels were affected by mitochondrial fragmentation induced by the knockdown of Cdh1. Although overall ATP levels were not remarkably different in any of the cell types tested (Figure 4-7A), the acute knockdown of Cdh1 in a G1/S-arrested Hela cell population drastically altered the profile of lipid-derived metabolites. Cdh1 knockdown cells showed a decrease in the abundance of most medium chain acylcarnitines and several long chain acylcarnitines, a profile that is consistent with a block in fatty acid β-oxidation. Many of these changes were attenuated by dual knock-down of Drp1 and Cdh1, suggesting that the β-oxidation defects produced by loss of Cdh1 correlated with Cdh1-mediated changes in Drp1 levels (Figure 4-7C). For a number of the metabolites measured, loss of Drp1 alone produced changes in levels opposite to loss of Cdh1, again suggesting that perturbation of Drp1 levels, with consequent alterations in mitochondria and peroxisomes (both are sites of β-oxidation where Drp-1 controls organelle division) could have marked effects on cellular metabolism.

Though we did not observe delays in cell cycle progression due to alterations in mitochondrial morphology during our limited time course, it is quite possible that the increased mitochondrial fragmentation we observed (due to loss of Cdh1) might have cumulative effects following several cell divisions. The persistently fragmented

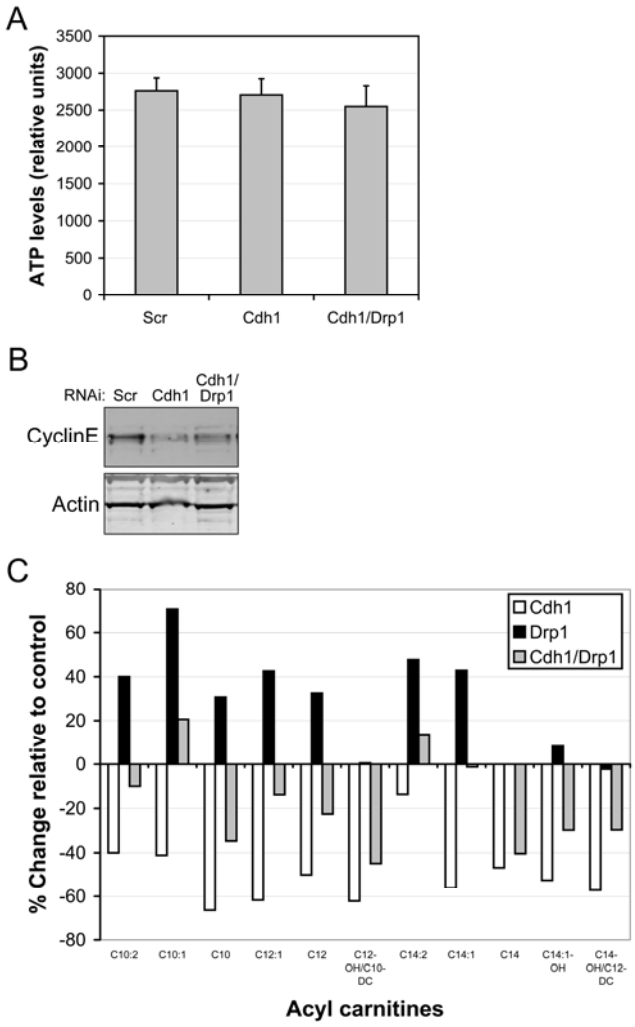

Figure 4-7: Cdh1 regulates Drp1-dependent changes in mitochondrial metabolism during interphase.

(A) ATP levels in siRNA-transfected Hela, arrested at the G1/S boundary. Equal cells numbers were harvested following the second thymidine incubation, and relative bioluminescence was measured for triplicate samples. (B) Cyclin E immunoblotting in siRNA-transfected, early G1-phase Hela cells. (C) Tandem mass spectrometry-based analysis of acylcarnitine species in G1/S-arrested Hela cells, transfected with the indicated siRNAs. Means of triplicate samples are shown as percent change relative to the scrambled RNAi control. Changes in medium and long chain acylcarnitines in the Cdh1-, Drp1-, and Cdh1/Drp1-RNAi cells are highlighted in the box.

mitochondrial morphology that results in the absence of Cdh1-mediated Drp1 degradation (and the subsequent decreases in cyclin E and alterations in lipid-derived metabolites) may have cumulative deleterious effects on mitochondrial function, comparable to the severe defects in cell growth, mitochondrial membrane potential and cellular respiration observed in cells deficient in the mitochondrial fusion proteins Mfn1 and Mfn2 (which display fragmented mitochondria due to a complete lack of mitochondrial fusion) (Chen et al., 2005).

4.3.1 Cdh1-dependent changes in mitochondrial metabolism

In our analysis of metabolic changes during interphase, we uncovered several additional alterations that occurred when Cdh1 was silenced by RNAi. Specifically, we found dramatic alterations in the acylcarnitine profiles, consistent with a total block in mitochondrial β-oxidation, and decreases in several essential amino acids and decreased glucose oxidation. Two recent reports have also implicated Cdh1 in metabolic signaling pathways. APC/C^{Cdh1} maintains the bioenergetic status of neurons through downregulation of a key glycolytic enzyme, and Akt-mediated phosphorylation of the APC/C^{Cdh1} substrate Skp2 impairs its degradation by the APC/C (Gao et al., 2009; Herrero-Mendez et al., 2009). These reports and our data suggest that Cdh1-mediated degradation may regulate multiple aspects of cellular metabolism during interphase, and further investigation into regulation of cellular metabolism by cell cycle proteins is an interesting new area of research.

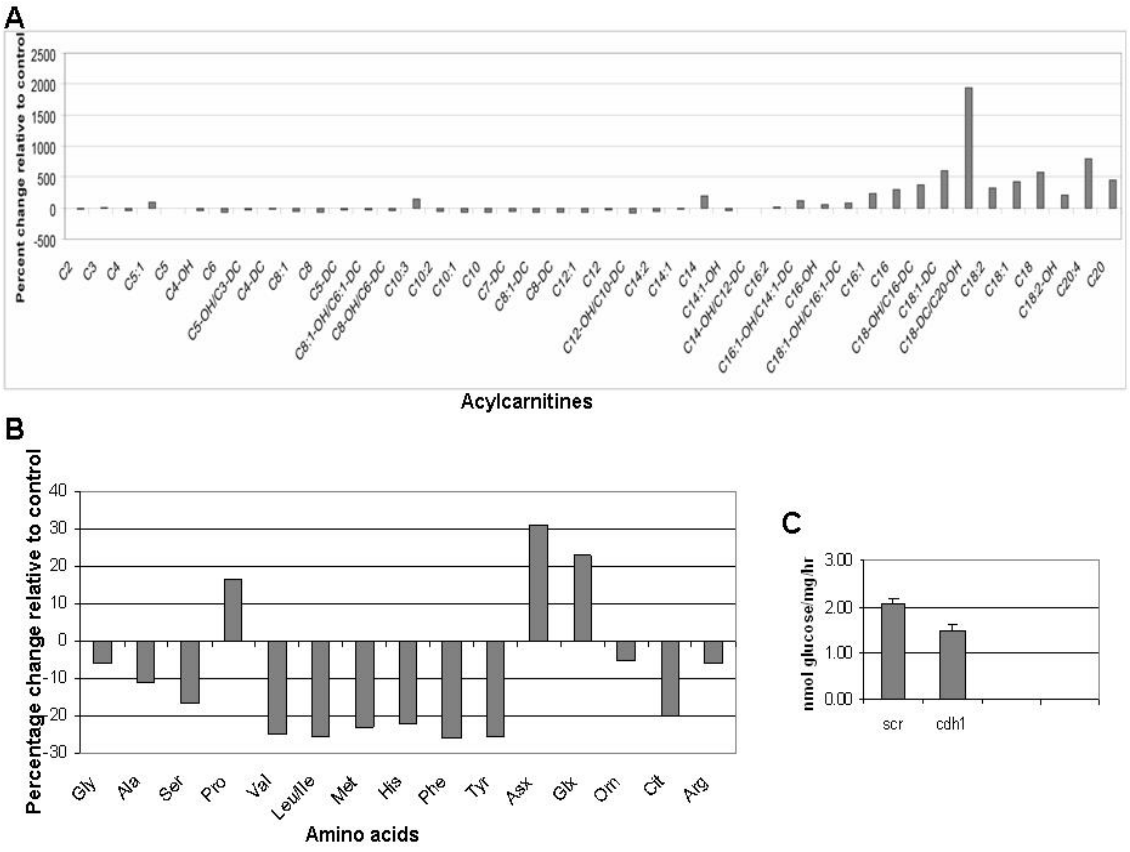

Figure 4-8: Cdh1-mediated metabolic changes during interphase.

Tandem mass spectrometry-based analysis of acylcarnitine species in G1/S-arrested Hela cells, transfected with the either Scr RNAi or Cdh1 RNAi. Means of triplicate samples are shown as percent change relative to the scrambled RNAi control. (B) Tandem mass spectrometry-based analysis of amino acids levels in G1/S-arrested Hela cells, shown as a percent change relative to the scrambled RNAi control. (C) Glucose oxidation assay of G1/S-arrested Hela cells, transfected with either Scr RNAi or Cdh1 RNAi.

Through the degradation of substrates such as the SCF component Skp2 and the inhibitor of DNA replication geminin, the APC/C^{Cdh1} complex regulates the proper timing of G1 and S phases. Here, we describe a novel role for APC/C^{Cdh1} in the degradation of the mitochondrial fission protein Drp1. APC/C^{Cdh1}-mediated Drp1 degradation is important for maintaining mitochondrial network formation and metabolic function during interphase, suggesting that APC/C^{Cdh1} complex may also regulate the distinct bioenergetic needs of a growing cell during synthetic phases of the cell cycle.

5. Conclusions and perspectives

Over 100 years ago, mitochondria were described as a collection of individual vesicles floating within the cell. Now, we understand that mitochondria form a highly dynamic reticulum, undergoing frequent fusion and fission events. Regulated changes in the morphology of the mitochondrial network occur in response to various cellular signals, and new evidence demonstrates that retrograde pathways relay signals from the mitochondria to the rest of the cell. This coordination between cellular and mitochondrial signaling pathways reveals a symbiotic relationship between mitochondria and eukaryotic cells that has been fine-tuned over billions of years.

Work from this dissertation furthers previous knowledge of how changes in the mitochondrial network are mediated at the molecular level. The data presented here further our understanding of how mitochondrial fission occurs during apoptosis and offer insight as to why this key event in programmed cell death has been conserved throughout evolution. Our characterization of Drp1 degradation by the APC/C^{Cdh1} complex helps to explain the changes in mitochondrial morphology and metabolism that occur during the cell cycle.

5.1 Reaper-mediated mitochondrial fragmentation during apoptosis

In Chapter 3, we describe Reaper-mediated mitochondrial fragmentation in mammalian cells as well as in the fly, *Drosophila melanogaster*. Though Reaper is a fly protein, its ability to stimulate mitochondrial cytochrome c release and caspase activation in vertebrate systems has been well characterized. In using a fly protein, we were able to examine the mechanism(s) by which mitochondrial fusion is inhibited during apoptosis in a simpler model system. Based on the conservation of the fission and fusion proteins throughout evolution, we hope to apply our findings of Reaper-mediated mitochondrial fission in the fly (and mammalian cells) in order to better understand the function of mitochondrial disruption and elimination in the vertebrate apoptotic pathway.

We observed that like Bax, Bak, Mfn2 , and Drp1, Reaper localizes to mitochondrial fission foci, and this finding suggests that Reaper may function with these proteins in the apoptotic fission process. We hypothesized that Reaper-induced mitochondrial fission results from either the activation of Drp1-mediated fission or via the inhibition of mitofusin-mediated mitochondrial fusion. Though we did not observe colocalization of Reaper with Drp1, we did observe striking colocalization of Reaper with both Mfn2 and the fly mitofusin dMFN. Additionally, we detected binding between Reaper and Mfn2 and dMFN in a number of different biochemical assays,

confirming that the colocalization we observed reflects an interaction between the proteins.

Mutational analyses revealed that the mitochondrial targeting of Reaper via its GH3 domain is essential for its colocalization with Mfn2 and its ability to induce mitochondrial fission. Though the GH3 domain was required, the IAP-Binding Motif (IBM) of Reaper was not; Reaper lacking the IBM domain (amino acids 1-15) was still capable of inducing mitochondrial fragmentation. This finding is consistent with previous reports of an additional, IAP-independent function of Reaper during apoptosis.

Importantly, we found that overexpression of dMFN suppresses Reaper-induced death in both wing discs and in fly S2 cells. The observation that overexpression of dMFN can suppress apoptosis downstream of Reaper suggests that active suppression of the fusion program occurs during programmed cell death. Together with the stimulation of Drp1-dependent fission during apoptosis, this inhibition of fusion may help tip the balance towards a fragmented mitochondrial network.

From our findings of Reaper-induced mitochondrial fission, we have insight into the function of mitochondrial fission during programmed cell death. In vertebrate systems which require the release of cytochrome c for apoptosis, fission seems to facilitate its timely release, as evidenced by the delays (or blocks) in cytochrome c release when Drp1 is inactivated (Abdelwahid et al., 2007; Ishihara et al., 2009; Wakabayashi et al., 2009; Youle and Karbowski, 2005). By contrast, in organisms like the worm and the

fly, where cytochrome c release is not an essential upstream event in the initiation of apoptosis, mitochondrial fission still occurs. In these organisms, we see evidence of the more primordial role of fission; perhaps mitochondrial fragmentation serves to inactivate mitochondrial metabolic function and/or to facilitate mitochondrial elimination. Through studying these organisms, we understand the full contribution of regulated mitochondrial fission to the apoptotic program, and we see how evolution may have modified this essential step in the development of the vertebrate cell death pathway.

5.2 Regulation of mitochondrial morphology and metabolism by APC/C^{Cdh1}

In Chapter 4, we describe a novel role for APC/C^{Cdh1} in the degradation of the mitochondrial fission protein Drp1. APC/C^{Cdh1}-mediated Drp1 degradation is important for maintaining mitochondrial network formation and metabolic function during interphase, suggesting that APC/C^{Cdh1} complex regulates the distinct bioenergetic needs of a growing cell during synthetic phases of the cell cycle.

We found that Drp1 levels change dramatically throughout the cell cycle. Drp1 levels are highest in mitosis and lowest during interphase, and this difference in protein levels correlates with the changes in morphology that we and others have observed (Barni et al., 1996; Margineantu et al., 2002; Taguchi et al., 2007). We observed that Drp1 was stable in mitotic cell lysates, whereas it was rapidly degraded in lysates prepared from G1 cells, suggesting that Drp1-directed degradation factors are active during G1.

Based on the timing of Drp1 degradation, we investigated the possibility that Drp1 is a substrate of the APC/C. We found that coexpression of Drp1 with Cdh1, but not Cdc20, caused its proteasome-dependent degradation. Furthermore, we detected binding between Drp1 and Cdh1 in cell lysates. We also noted that overexpression of Cdh1 alone, in asynchronous cells, resulted in dramatic changes in the mitochondrial network. Specifically, in cells expressing Cdh1, mitochondria looked to be more interconnected, a morphology that is consistent with decreased mitochondrial fission.

We examined the Drp1 amino acid sequence and found multiple potential degrons, including nine minimal RxxL motifs as well as non-canonical motifs such as a GxEN box and an O-Box. Analysis revealed that mutation of the cyclin B type D-Box (R-X-X-L-X-X-X-X-N/D/E) attenuated Cdh-1 dependent ubiquitylation and increased protein stability in a number of different cell-based assays. Though the D-Box mutant was more stable that the wild-type Drp1 protein, overexpression of either the wild-type or the D-Box mutant in early G1 cells yielded the same fragmented morphology.

To investigate the function of Cdh1-mediated Drp1 degradation in a cleaner system, we utilized cells which had decreased Cdh1 (due to genetic deletion in the MEFs or RNAi-mediated silencing in Hela cells). In the absence of Cdh1, we observed increased Drp1 levels and decreased Drp1 ubiquitylation. Moreover, we saw dramatic alterations in mitochondrial morphology in the absence of Cdh1: cells lacking Cdh1 had fragmented mitochondria during interphase, and this change was attenuated with dual

knockdown of both Cdh1 and Drp1. These data are consistent with a requirement for Cdh1-mediated Drp1 degradation in maintaining an interconnected mitochondrial morphology during interphase.

We also observed that inhibition of Cdh1-mediated Drp1 degradation resulted in metabolic alterations during interphase. Though ATP levels were unchanged, inhibition of Cdh1 led to decreased cyclin E levels and a dramatic difference in acylcarnitine profiles. These data support a role for Cdh1 in maintaining proper mitochondrial morphology and metabolic function during interphase.

Here, we describe a new role for APC/C^{Cdh1} in the regulation of mitochondrial morphology and metabolism through degradation of the mitochondrial fission protein Drp1. Together, these data suggest that the APC/C^{Cdh1} complex may regulate the distinct bioenergetic demands of an interphase cell, and they provide additional evidence of a growing link between the coordination of cellular metabolism and the cell division program.

5.3 Mitochondrial dynamics in health and disease

Mitochondrial dynamics play an integral role in cellular function. Regulated mitochondrial fission and fusion events are important for in a variety of cellular processes, ranging from apoptosis and cell division to the maintenance of calcium homeostasis and the elimination of dysfunctional mitochondria. Based on the essential

role of mitochondrial fission and fusion, it is not surprising that defects in mitochondrial dynamics can have negative consequences for the health of the organism.

5.3.1 Mitochondrial dynamics in development

Genetic deletion of Mfn1, Mfn2, or Drp1 results in embryonic lethality, underscoring the importance of mitochondrial dynamics during embryonic development. Interestingly, the cause of lethality in these knockout animals (as well as in the Cdh1 knockout mouse) is defective placental development due to disruption of the placental trophoblast giant cell layer (Chen et al., 2005; Chen et al., 2007a; Garcia-Higuera et al., 2008; Ishihara et al., 2009; Li et al., 2008; Wakabayashi et al., 2009). Trophoblast giant cells are large, polyploid cells generated through the process of endoreduplication (in which DNA is replicated repeatedly without an intervening cytokinesis) (Cross, 2000). Giant cells are highly metabolically active, and it is possible that aberrant mitochondrial dynamics and/or alterations in mitochondrial metabolism contribute to placental abnormality in all of these lethal phenotypes.

In addition to their essential functions in placental development, components of the mitochondrial dynamics machinery may have important roles in other aspects of embryonic development. Morphological changes have been observed in mitochondria during the first embryonic divisions. As cells in the developing embryo begin to produce ATP and consume oxygen through aerobic respiration, mitochondrial elongation occurs and mitochondrial cristae number increases (May-Panloup et al., 2007; Sathananthan

and Trounson, 2000). These marked changes in mitochondria morphology and metabolism suggest that mitochondrial fusion and fission processes may serve key functions in mediating metabolic transitions within the developing embryo.

In the context of experimental evidence supporting protective roles for fission and fusion in guarding against mitochondrial dysfunction (through the complementation of mtDNA gene products and matrix components as well as the role for mitochondrial dynamics in sorting and eliminating dysfunctional mitochondrial through mitophagy), it is interesting to consider a possible role for regulated mitochondrial fusion and fission during the processes of embryonic oogenesis and folliculogenesis, during which mitochondrial selection events occur. The first event has been described as a "filter for purifying selection," in which severe mtDNA mutants (such as those severely affecting OXPHOS or proofreading by the mtDNA polymerase) are rapidly eliminated in the germline (Fan et al., 2008; Stewart et al., 2008a; Stewart et al., 2008b). In addition to this selection event, data support a mitochondrial genetic bottleneck in which a random subpopulation of mtDNAs are replicated during postnatal folliculogenesis (Wai et al., 2008). Based on the protective roles for mitochondrial fission and fusion in preventing mitochondrial dysfunction, it would be interesting to examine the contribution of mitochondrial dynamics to the transmission of mtDNA in the female germline. We hypothesize that impairments in fission/fusion (and subsequent failure to eliminate dysfunctional mitochondria or to complement mutant mtDNA) might impede

the "purifying selection filter," resulting in deleterious effects on fitness or viability for future generations.

Though the requirement for mitochondrial dynamics in development is well established, a new role for mitochondrial fission/fusion dynamics in pluripotent mouse embryonic stem cells (ESCs) was recently reported (Todd et al., 2010). In the absence of the stem cell growth factor, Gfer, Drp1 protein levels are elevated and excessive fragmentation and mitophagy occur. Modulation of Drp1 levels by Gfer preserves mitochondrial morphology and function and maintains pluripotency of the mouse ESC population. Understanding the regulation of mitochondrial dynamics in stem cell populations is a very interesting new area, deserving of further study.

5.3.2 Mitochondrial dynamics in neuronal development and neurodegeneration

Due to their unique energetic requirements, cells of the nervous system appear to be particularly sensitive to defects in mitochondrial function, and mitochondrial fission and fusion are required both during nervous system development and for homeostatic maintenance in the adult organism. Mfn2 conditional KO mice demonstrated a requirement for Mfn2 in postnatal development of the cerebellum; massive degeneration of the Purkinje cells occurs in Mfn2 null cerebella, resulting in severe postural and movement defects in the mice (Chen et al., 2007a). In the fly, mutations in Drp1 (the *Fratboy* mutant) are semilethal, and the survivors experience neurodegeneration and lack coordination, like the Mfn2 conditional knock-out mice (Verstreken et al., 2005).

Drp1 -/- mice also have defects in the development of the cerebellum as well as the forebrain, and further characterization of the Drp1 null forebrain revealed striking defects in synapse formation (Wakabayashi et al., 2009).

The distribution of mitochondria into synaptic regions of neurons supplies the cells with ATP to drive the energy-dependent process of synaptic transmission. In CMT-2A, pathogenic mutants of Mfn2 induce abnormal mitochondrial clustering in neurons and diminished mitochondrial transport into axons (Baloh et al., 2007). These data and other experimental evidence highlight the importance of the fission/fusion machinery in synapse formation and maintenance, as impaired mitochondrial fission and fusion lead to defects in the trafficking and distribution of mitochondria into synaptic regions of neurons (reviewed in Chen and Chan, 2009; McBride et al., 2006).

The APC/C coactivator Cdh1 has also been shown regulate synaptic growth and transmission (Gieffers et al., 1999; Konishi et al., 2004; van Roessel et al., 2004), and Cdh1 null mice show severe nervous system defects (Garcia-Higuera et al., 2008; Li et al., 2008). In light of our characterization of Drp1 as an APC/C^{Cdh1} substrate, it would be interesting to examine if and how this regulatory axis might function in neurons.

In addition to DOA and CMT-2A diseases, with underlying mutations in Opa1 and Mfn2 respectively, there is evidence implicating defective mitochondrial dynamics in at least two other neurodegenerative disorders: Parkinson's and Alzheimer's diseases. Defects in the elimination of dysfunctional mitochondria due to loss-of-function

mutations in the mitochondrial kinase PINK1 and the E3 ubiquitin ligase Parkin are implicated in the pathogenesis of familial forms of Parkinson's disease, and experimental evidence supports a role for the mitochondrial fission/fusion machinery in Parkin-mediated mitochondrial clearance (Deng et al., 2008; Narendra et al., 2008; Poole et al., 2008; Vives-Bauza et al., 2010; Vives-Bauza et al., 2010; Ziviani et al., 2010). In Alzheimer's disease, reduced metabolism in the brain one of most well characterized abnormalities, although increased oxidative stress and alterations in calcium homeostasis in neurons have also been reported (reviewed in Wang et al., 2009). Mitochondrial fission and fusion contribute to the homeostatic maintenance of many of the cellular processes that are dysfunctional in Alzheimer's disease; for example, both Mfn2 and Drp1 have important roles in mitochondrial calcium buffering (de Brito and Scorrano, 2008; Szabadkai et al., 2004). A more direct link between disease pathogenesis and aberrant morphology was recently reported; Cho et al. found that S-nitrosylation of Drp1 (generating SNO-Drp1) stimulates mitochondrial fission, and SNO-Drp1 is increased in brains of Alzheimer's patients (Cho et al., 2009). Thus, aberrant mitochondrial fission may contribute to neuronal injury and subsequent neurodegeneration in Alzheimer's and Parkinson's diseases.

5.3.3 Mitochondrial dynamics and metabolism

Our finding that inhibition of Cdh1-mediated degradation of Drp1 during interphase prevents cyclin E accumulation and alters the profile of lipid-derived

metabolites is part of growing evidence of a link between cellular proliferation and mitochondrial metabolism. Evidence from multiple research groups suggests a "metabolic checkpoint" at the G1/S boundary in which several signaling pathways (including AMP-Activated protein kinase (AMPK)-induced p53 activation as well as ROS-mediated upregulation of a fly CKI homologue) converge to modulate cyclin E levels and activity (Jones et al., 2005; Mandal et al., 2005; Mitra et al., 2009; Owusu-Ansah et al., 2008; Mandal et al., 2010). Together, these data highlight an exciting new area of research on the contribution of mitochondrial metabolism and network status to cell cycle progression.

In our analysis of Cdh1 function during interphase, we uncovered several metabolic alterations that occurred when Cdh1 was silenced by RNAi. Specifically, we found dramatic alterations in the acylcarnitine profiles, consistent with a total block in mitochondrial β-oxidation, and decreases in several essential amino acids and decreased glucose oxidation. These data, together with two recently published reports implicating Cdh1 function in metabolic signaling pathways, suggest that the role of Cdh1 as a "master regulator" of G0/G1 phases may also include the regulation of cellular metabolism during interphase (Gao et al., 2009; Herrero-Mendez et al., 2009; Skaar and Pagano, 2008). Cdh1-mediated degradation of Drp1 accounted for a subset of the alterations in acylcarnities that we observed; it is likely that additional (Drp1-

independent) Cdh1-mediated metabolic changes exist, and like Drp1, these changes may be mediated through degradation of the metabolic substrates by the APC/C^{Cdh1} complex.

Based on the correlation between mitochondrial network status and the type of cellular energy substrate utilized, with oxidative substrates yielding a more thin, branched mitochondrial network (that is more efficient in mitochondrial fusion) than glycolytic substrates (Benard et al., 2007; Benard and Rossignol, 2008; Jakobs et al., 2003; Meeusen et al., 2004; Rossignol et al., 2004), it would be interesting to determine whether this relationship functions in both directions; i.e. how does changing morphology alter metabolism? Our findings that levels of certain acylcarnitine species were correlated with either a fragmented or fused mitochondrial network suggest that the relationship between morphology and metabolic output may be bi-directional. To test this aspect of mitochondrial dynamics, we could experimentally manipulate mitochondrial morphology (through RNAi or chemical inhibition of the fission/fusion machinery) and then test for alterations in metabolomic profiles.

In addition to the evidence supporting a bi-directional relationship between mitochondrial metabolic function and cellular function, there is evidence supporting a link between morphology and metabolism in disease pathogenesis. For example, defects in mitochondrial network formation were observed in obese, insulin-resistant patients, and these defects were correlated with decreased Mfn2 expression in muscle. When the patients underwent weight-loss surgery, improvements in insulin sensitivity and

glucose oxidation occurred, and these changes were correlated with increased mitochondrial network formation and increases in Mfn2 expression (Bach et al., 2005; Bach et al., 2003; Pich et al., 2005; Zorzano, 2009). Additionally, in cell models of type 2 diabetes, mitochondria fission occurs following exposure to high glucose and fat levels (Molina et al., 2009; Yu et al., 2006a). Together with the metabolic abnormalities observed in the patient with a Drp1 mutation (Waterham et al., 2007), these observations reveal an important link between mitochondrial morphology and metabolic efficiency.

5.3.4 Mitochondrial dynamics in aging

Proteins of the mitochondrial fission and fusion machinery have an integral role in mitochondrial functions like metabolism and apoptotic regulation. In several cases, a direct link exists between the fission/fusion machinery and mitochondrial metabolic outputs, like ROS, a major source of oxidative damage within the cell (Tang et al., 2009; Yarosh et al., 2008; Yu et al., 2006a). Acute and chronic ROS damage can result in apoptosis, with the latter thought to be a cause for the decline of cellular function over time, according to mitochondrial theories of aging (Wallace, 2005). New experimental evidence supports a role for the fission/fusion machinery in this decline. In Drosophila models of DOA, work from the lab of Dr. Huang has demonstrated that mutations in Opa1 cause an increase in ROS, associated with damage and loss of the cone and pigment cells of the eye (Yarosh et al., 2008). The findings in the eye model were later extended to the whole fly; Dr. Huang and colleagues reported that heterozygous

mutation in Opa1 increases ROS levels, increases susceptibility to oxidative stress and shortens the lifespan of flies carrying the mutation (Tang et al., 2009).

Consistent with these reports of impaired Opa1-mediated membrane fusion shortening lifespan, there is experimental evidence that reducing mitochondrial fission actually increases lifespan. In two different fungal aging models, deletion of the *Dnm1* gene (the yeast Drp1 orthologue) slowed aging without impairing fitness and fertility and extended the "health span," the healthy period of time in the life cycle of the organisms (Scheckhuber et al., 2007). Collectively, these data demonstrate that the mitochondrial fission/fusion machinery functions in the aging process.

In this regard, it is interesting to consider how the relationship between mitochondrial dynamics and mitochondrial metabolic changes might also contribute to aging. A diet high in glucose and fat inhibits mitochondrial fusion and stimulates mitochondrial fission (Molina et al., 2009; Zorzano, 2009), and significant experimental evidence supports caloric restriction as a means of extending lifespan (Masoro, 2009; Piper and Bartke, 2008). With such a restricted diet, we would hypothesize that diet-induced mitochondrial fission is suppressed. Based on the fungal model in which decreased mitochondrial fission slows aging, the prevention of excess mitochondrial fission may provide a possible a mechanism by which caloric restriction increases lifespan.

5.4 Concluding remarks

The data presented in this dissertation have advanced our understanding of how mitochondrial fragmentation is regulated during key cellular processes like apoptosis and cell division. During cell death, regulated mitochondrial fission is essential for progression of the apoptotic program. In the cell cycle, mitochondrial fragmentation ensures the equal distribution of mitochondria into daughter cells, and cell cycle-dependent alterations in mitochondrial dynamics suit the distinct bioenergetic needs of a proliferating cell.

Though mitochondrial dynamics is a relatively new field (with the first reports emerging in the late 1990s), rapid progress has been made in understanding the complex regulation of mitochondrial fission and fusion at the molecular level. Identifying and characterizing the molecular mechanisms regulating mitochondrial dynamics will help us to better understand and predict how disruption of the balance between mitochondrial fission and fusion may contribute to a variety of disorders and natural processes, ranging from diabetes, to neurodegeneration, and even to human aging.

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Biography

Sarah Rapisardo Horn

Born to Jan and Thomas Rapisardo, on October 2, 1978 in Milwaukee, WI
Married to Patrick E. Horn, on October 6, 2001 in Sullivan's Island, SC

Education

Ph.D. in Molecular Cancer Biology, Certificate in Cell and Molecular Biology
Duke University, Durham, NC, May 2010

B.A. in Biology and English with Highest Distinction
UNC-Chapel Hill, Chapel Hill, NC, May 2001

International Baccalaureate Diploma
Myers Park High School, Charlotte, NC, June 1997

Publications

Horn SR, Thomenius MJ, Wu JQ, An J, Ilkayeva OR, Segear EL, Coloff JL, Freel CD, Rathmell JC, Newgard CB, and Sally Kornbluth. Regulation of mitochondrial morphology by APC/C^{Cdh1}-mediated control of Drp1 stability. *In Revision*.

Todd LR, Damin, MN, Kokubu M, Gomathinayagam R, **Horn SR**, Means AR, Sankar U. Growth factor *erv1*-like modulates Drp1 to preserve mitochondrial dynamics and function in mouse embryonic stem cells. *Mol. Biol. Cell*. 2010. E-pub ahead of print.

Wieman HL, **Horn SR**, Jacobs SR, Altman BJ, Kornbluth S, Rathmell JC. An essential role for the Glut1 PDZ-binding motif in growth factor regulation of Glut1 degradation and trafficking. *Biochem J*. 2009. 418(2):345-67.

Freel CD, Richardson DA, Thomenius MJ, Gan EC, **Horn SR**, Olson MR, Kornbluth S. Mitochondrial localization of reaper to promote IAP degradation conferred by GH3 domain-lipid interactions. *J Biol Chem*. 2008. 283(1):367-79.

Lam SY, **Horn SR**, Radford SJ, Housworth EA, Stahl FW, and Copenhaver GP. Crossover interference on nucleolus organizing region-bearing chromosomes in Arabidopsis. *Genetics*. 2005. 170:807-12.

Honors and Awards

2009: **Invited speaker:** Keystone Joint Symposium on Mitochondrial Dynamics and Physiology; March, 2009

2009: **Conference Travel Fellowship**, Duke University Graduate School, 2009

2009: **Speaker:** Duke Department of Pharmacology and Cancer Biology Annual Retreat; September, 2009

2005: **Honorable Mention**: NSF Graduate Research Fellowship

2004-2006: **NIH Cell and Molecular Biology Training Fellow**, Duke University

2000-present: **Member,** Phi Beta Kappa