NORTHWESTERN UNIVERSITY

Investigating the Mechanisms of Mmr1 in Mitochondrial and mtDNA Inheritance

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Field of Interdisciplinary Biological Sciences

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EVANSTON, ILLINOIS

September 2020

ABSTRACT

Proper partitioning of mitochondria and mtDNA is critical for cellular health. Investigations into mitochondrial inheritance, specifically how mtDNA inheritance is coupled with the inheritance mitochondrial compartment, are still in the early stages. We use budding yeast as a model polarized cell system to study a mitochondrial Myo2-adaptor protein, Mmr1, in order to understand the mechanisms by which cells partition mitochondria prior to cell division.

Mitochondrial transport and anchoring mechanisms work in concert to position mitochondria and ensure proper mitochondrial inheritance. In budding yeast, Mmr1 functions as a mitochondrial adaptor for Myo2 to facilitate actin-based transport of mitochondria to the bud. Post-transport, Mmr1 is proposed to anchor mitochondria at the bud tip. More importantly, Mmr1 has been suggested to be involved in the asymmetric partitioning of functioning mitochondria and the age asymmetry of budding yeast. Despite its importance, the molecular basis and mechanism of Mmr1-dependent mitochondrial inheritance is poorly understood. Our in vitro phospholipid binding assays indicate Mmr1 can directly interact with phospholipid membranes. Through structure-function studies we identified an unpredicted membrane-binding domain composed of amino acids 76-195 that is both necessary and sufficient for Mmr1 to interact with mitochondria in vivo and liposomes in vitro. In addition, our structure-function analyses indicate that the coiled-coil domain of Mmr1 is necessary and sufficient for Mmr1 self-interaction and facilitates the polarized localization of the protein. Disrupting either the Mmr1-membrane interaction or Mmr1 self-interaction leads to defects in mitochondrial inheritance. Therefore, direct membrane binding and self-interaction are necessary for Mmr1 function in mitochondrial inheritance and are utilized as a means to spatially and temporally regulate mitochondrial positioning. In addition to its role in the inheritance of the mitochondrial

compartment, we find that Mmr1 plays a role in maintaining mtDNA integrity over generations and this role for Mmr1 is likely linked to its role in mitochondrial transport. Overall, these findings expand our knowledge of mitochondrial and mtDNA inheritance and contribute to the understanding of mitochondrial partitioning in asymmetrically dividing cells.

Acknowledgements

I would like to thank my advisor, Laura Lackner. You have been an extraordinary mentor and for me personally a "lab mom". As a mentor, your enthusiasm and knowledge for science has helped me grow tremendously as a scientist. As a "lab mom", your selflessness and desire to see us all of us succeed has made our lab feel like a family. Your "lab children" come in a diverse bunch and thank you for loving us all and giving us our individual attentions. As the proverb states, "It takes a village to raise a child". I want to say thank you for creating that village.

I would like to thank all the Lackner Lab members for being great colleagues and friends. Thank you for all these years of support, care, and encouragement. You guys have created some of the most valuable moments in my life. To Holly, Amy, and Lauren – thank you for welcoming me into the lab from the start. You guys created the lab environment that has shaped the last six years of my life and made it a place I wanted to come every day. Seeing your interaction with each other and with Laura was a key driver in my decision to join the lab. To Heidi and Antoineen – thank you for joining the lab and continuing to make our lab great. Thank you for you making every day enjoyable for the past how many years and thank you for sharing some of my precious memories. To Clare, Brett, Conor and Michael – thank you for joining the lab and believing in us. I know you will continue to make the lab a great environment for future students. To Jenni – thank you for all your scientific and life tips. I enjoy having you in the lab and am sad that we only overlapped for a short time.

I would like to thank my family for all your support. With very little knowledge of what it meant to pursue a PhD, you packed me up and helped me move to Chicago and have continued to provide your support throughout my graduate school experience. When I said I had no idea

when I would graduate, probably not for 5-6 years and I would only make enough money to support myself, you guys said, we came to America because we wanted you to pursue whatever you desired and we don't understand this, but we know you will make the best decision for you.

I would also like to thank my friends for bringing joy to my life outside of the lab. To Jasmine and Takuma - thank you for your company all these years together and thank you in advance for the many years of friendship to come. Special thanks to college friends, Valinda, OiYee, Mary, and Jenny - you guys have always provided your support and encouragement. Thank you for always making sure I know no matter what happens in life, I always have you guys to fall back on.

I would also like to thank my friends who I met outside of the graduate program mostly through the Northwestern Chinese Reading Club and the Northwestern Chinese Christian Fellowship. Knowing you guys helped me think about my own identity and my position in the world. Thank you for being good listeners and thank you for all the good discussions we had together.

Thank you to my committee members, Jason Brickner, Sadie Wignall, and Xiaomin Bao. Over the years, your suggestions and support have been valuable for the advancement of my project and me as a scientist.

I would like to express my gratitude toward the Northwestern community. Thank you to the Cell Biology Supergroup for providing a welcoming and constructive environment to share my research. Thank you to Jessica Hornick and the staff in BIF for training me on the microscopes and providing me with dedicated support when needed. Thank you to Deborah Klos Dehring, Cathy Prullage, and the Molecular Biosciences Office for addressing my many questions along the way. Lastly, thank you to the Biophysics Training Grant at Northwestern for

funding this research project. The chances to present on multiple occasions and the feedback I received during these interactions, helped me to grow as a scientist.

Abbreviations

AID, auxin inducible degron

ATP, adenosine triphosphate

CC, coiled-coil

CL, cardiolipin

cER, cortical endoplasmic reticulum

ER, endoplasmic reticulum

ERMES, ER-mitochondria encounter structure

ETC, electron transport chain

MECA, mitochondria-ER-cortex anchor

mtDNA, mitochondrial genome

mtDNA-CN, mitochondrial DNA copy number

OXPHOS, oxidative phosphorylation

PA, phosphatidic acid

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

PGC, primordial germ cells

PI, phosphatidylinositol

$PI_{4,5}P_2$, phosphatidylinositol 4,5-bisphosphate

PM, plasma membrane

PS, phosphatidylserine

PTM, post-translational modification

ROS, reactive oxygen species

S. cerevisiae, *Saccharomyces cerevisiae*

yEGFP, yeast-enhanced GFP

yEmCherry, yeast-enhanced mCherry

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Chapter 1:

Introduction

1.A Introduction to organelles and organelle partitioning

Eukaryotic cells are compartmentalized into membrane-bound organelles that carry out distinct and essential cellular functions. Many of these organelles are distributed throughout the cell in order to meet cellular needs. During cell division, most organelles need to be inherited by both daughter cells in order for each cell to maintain viability, as most membrane bound organelles, such as mitochondria and the endoplasmic reticulum (ER), cannot be generated de novo. There are two general models for organelle inheritance: passive and active. The passive segregation model proposes a more stochastic organelle distribution mechanism; while active segregation of organelles typically requires motor proteins that bind to the organelles and move them along cytoskeletal filaments such as microtubules and actin filaments.

In higher eukaryotes, such as mammalian cells, microtubule-based transport serves as the primary active transport mechanism for organelles. On the other hand, *Saccharomyces cerevisiae,* also known as the budding yeast, primarily utilizes actin-based transport for the transport of organelles. Although these two types of eukaryotes primarily utilize different filaments to transport their organelles, both require a "track" to direct organelle movement, and a "motor" to power this movement. Given the diversity of membrane-bound organelles and the importance of their inheritance, the correct transport of organelles to a specific location at a precise time is highly regulated (Fagarasanu e*t al*., 2006, Jin *et al*., 2015, Jin *et al*., 2014, Peng *et al*., 2008, Yau *et al*., 2014).

In budding yeast, most organelles rely on a class V myosin motor-dependent mode of inheritance. Yeast have two class V myosin motors, Myo2 and Myo4. Both of these class V myosin motors utilize actin cables for transport of organelles throughout the cell (Fagarasanu *et al*., 2006, Tang *et al*., 2008, Fortsch *et al*., 2011, Valiathan *et al*., 2008). The inheritance of

organelles such as mitochondria, ER, vacuoles, peroxisomes, and secretory vesicles is Myo2-dependent. Other organelles such as cortical (cER) require Myo4 for inheritance.

Since most organelles are inherited via Myo2, the competition between organelles associating with Myo2 must be tightly regulated. This regulation is facilitated by organelle-specific Myo2 adaptors that link organelles to their motor proteins, which then traffic the organelles to their destined locations. Mmr1 and Ypt11, Vac17, Inp2, and Sec4 are Myo2 adaptors for mitochondria, vacuoles, peroxisomes, and secretory vesicles, respectively (Hammer *et al*., 2011). These adaptor proteins contain Myo2 binding domains that interact with Myo2 at one of two overlapping sites on the Myo2 cargo binding domain. The mitochondrial adaptor Mmr1 and vacuolar adaptor Vac17 bind to the same region on Myo2, and the other mitochondrial adaptor Ypt11, the spindle positioning adaptor Kar9, and the peroxisome adaptor Inp2 bind to the other region on Myo2 (Eves *et al*., 2012). The binding of adaptors to the same region on Myo2 results in the competitive inheritance of their cognate organelles. For instance, Mmr1 and Vac17 compete for access to Myo2; therefore, the inheritance of mitochondria and vacuoles are in competition with each other. Altering the expression of either adaptor can affect the inheritance of both mitochondria and vacuoles (Eves *et al*., 2012). However, Mmr1 and Ypt11 interact with two distinct adaptor-binding regions on Myo2 and function in parallel pathways to transport mitochondria, as the double deletion of *MMR1* and *YPT11* has an additive effect on mitochondrial inheritance (Frederick *et al*., 2008).

This active mode of organelle inheritance is critical for asymmetric cell division, where cell division results in two cells that are not identical to each other. In yeast, damaged organelles, such as damaged mitochondria and vacuoles, are retained in the mother and higher functioning organelles are transported into the daughter (Higuchi-Sanabria *et al*., 2014, Smyth *et al*., 2015).

This asymmetric cell division ensures the daughter cell restores its replicative potential and is "born young" despite the condition of the mother cell (Higuchi-Sanabria *et al*., 2014). Myo2-dependent organelle inheritance has been suggested to play a key role in the asymmetric division of yeast cells by selectively transporting higher functioning organelles into the new daughter cell. Specifically, studies suggest mitochondrial transport and positioning mechanisms ensure mitochondria are differentially partitioned during cell division and affect the cytosolic and age asymmetry of the two resulting cells. In addition, recent studies have begun to examine the inheritance of mitochondrial DNA (mtDNA) and its role in asymmetric inheritance of mitochondria. My work provides insight into Mmr1-dependent mitochondrial inheritance. Specifically, I dissected the molecular mechanism and regions of Mmr1 necessary for Mmr1-dependent mitochondrial inheritance. My data also suggest Mmr1 plays a role in maintaining mtDNA integrity during cell division. Thus, my work contributed to the understanding of mitochondrial and mtDNA inheritance during asymmetric cell division.

1.B Mitochondria

Mitochondria are organelles found in most eukaryotic organisms. According to the endosymbiosis theory, mitochondria descended from a special class of bacteria, α-proteobacteria, which were engulfed by an early eukaryotic cell about two billion years ago (Margulis *et al*., 1970). It is believed that for this reason mitochondria are double membrane bound organelles. The two mitochondrial membranes encapsulate two distinct compartments that carry out specialized functions, the intermembrane space and the matrix. The intermembrane space lies between the mitochondrial inner and outer membranes, while the matrix space is enclosed by the inner membrane (Figure 1.1). This compartmentalization is essential for

mitochondrial function (Friedman *et al*., 2014). Inside the matrix space, mitochondria store their own genome, as well as transcriptional and translational machinery. Over the course of evolution, some mitochondrial genes encoded by the ancestral bacterial genome were transferred to the nuclear genome, and mitochondria evolved to perform multiple functions that are essential for a eukaryotic cell.

1.B.1 Mitochondrial DNA (mtDNA)

Mitochondria contain their own genome, the mitochondrial DNA (mtDNA), which is located in the mitochondrial matrix. Although most genes were lost to the nuclear genome, mtDNA still encodes some essential subunits of the oxidative phosphorylation (OXPHOS) pathway that are required for energy production, in the form of adenosine triphosphate (ATP), via cellular respiration. Although the exact number of genes found in mtDNA varies among species, all mtDNA encode essential proteins of OXPHOS complexes, and ribosomal RNA and transfer RNAs that are required for mitochondrial protein translation. Specifically, mtDNA in the yeast *S. cerevisiae* encodes seven essential proteins of the OXPHOS complexes, including subunits of ATP synthase (Atp6, Atp8, and Atp9), units of the cytochrome c oxidase (Cox1, Cox2, and Cox3) and apocytochrome b (Cob) (Freel *et al*., 2015). mtDNA also encodes one RNA subunit of RNAse P, one ribosomal protein, two ribosomal RNAs, and 24 transfer RNAs, all of which are required for mitochondrial protein translation (Malina *et al*., 2018, Aretz *et al*., 2020). While the nuclear genome is organized by wrapping around histones and compacting into chromatin, mtDNA is packaged into nucleoprotein complexes, which include mtDNA and proteins, termed nucleoids (Lipinski *et al*., 2010). The protein components of mitochondrial

DNA nucleoids are encoded by the nuclear genome and are responsible for the maintenance and expression of mtDNA.

1.B.2 Mitochondrial DNA copy number (mtDNA-CN)

In most eukaryotes, mtDNA is present in multiple copies (Osman *et al.,* 2015). The exact copy number of mtDNA can vary dramatically depending on the species and cell type. For example, in yeast mtDNA is maintained at ~50-200 copies, while in a human oocytes, mtDNA is maintained at 200,000 copies (Osman *et al*., 2015, Kasashima *et al*., 2014). In yeast cells, each nucleoid contains a range of 1-10 mtDNA copies (Chen *et al*., 2005). In mammalian cells, recent super-resolution microscopy experiments showed mitochondrial nucleoids contain only a single copy of mtDNA (Kukat *et al*., 2011).

In addition to mtDNA-CN varying across different cell types, in yeast, mtDNA-CN can also vary based on cell volume and growth condition. Studies have shown mtDNA-CN correlates linearly with the length of the mitochondria network (Osman *et al*., 2015). The length of the mitochondrial network also correlates with cell volume (Rafelski *et al*., 2012). Together, this suggests that mtDNA-CN correlates linearly with cell size. Growth conditions alter mitochondrial network volume resulting in altered mtDNA-CN. Yeast cells grown in non-fermentable carbon sources have a greater mitochondrial network volume. Since mtDNA-CN positively correlates with mitochondrial volume, mtDNA-CN is thought to scale accordingly and increase in non-fermentable conditions.

Although mtDNA-CN varies across different cell types and growth conditions, the copy number for a specific cell type under a specific growth condition is highly regulated. Altered mtDNA-CN has been increasingly used to assess the function of mitochondria. Several studies

have shown higher levels of mtDNA are associated with a decrease in neurodegenerative diseases and loss of mtDNA often causes defects in mitochondrial function (Filograna *et al*., 2019, Li *et al*., 2019). Although the maintenance of mitochondrial DNA-CN throughout the mitochondrial network is essential to meet the energy needs of the cell, the cellular mechanisms that regulate mtDNA-CN remain poorly understood.

1.B.3 Mitochondrial DNA mutations

The mitochondrial genome has about a 100-fold higher mutation rate than the nuclear genome due to lower mitochondrial DNA polymerase fidelity (Baile *et al*., 2013). Specifically, in mammalian cells, one study suggests that mitochondria may have a nucleotide imbalance that leads to a decrease in DNA polymerase gamma fidelity (Song *et al*., 2005). In yeast, mutations of the mitochondrial DNA Polymerase, *MIP1*, gene lead to an increase in the mtDNA mutation rate (Baruffini *et al*., 2007). In addition, mitochondrial produced reactive oxygen species (ROS) can damage mtDNA and cause a higher mutation rate (Murphy *et al*., 2009).

These mutations lead to a heterogeneous population of mtDNA within the same cell, meaning cells contain a variable ratio of mutated and wildtype mtDNA. As a result, mitochondria are considered heteroplasmic. In mammalian cells, mitochondria remain heteroplasmic, and mutations in mtDNA often do not result in noticeable phenotypes until a critical number of dysfunctional copies of mtDNA is reached because the remaining wildtype mtDNA copies are able to complement for the defect. This is known as the threshold effect. However, in yeast, mitochondria become homoplasmic within a few generations due to high recombination rates, allowing the dysfunctional mtDNA to be propagated throughout the mitochondrial network (Baile *et al*., 2013). Therefore, being able to select for and transport

functional mtDNA has been suggested to be critical to maintaining functional mtDNA for generations.

Mutations in mtDNA may cause respiration complex dysfunction, which leads to defects in energy production and the production of more ROS (Baile *et al*., 2013). Due to the importance of mtDNA in energy production, mutations in mtDNA that result in respiratory deficiencies are associated with a variety of human diseases, including neurodegenerative and metabolic diseases and various types of cancer (Friedman *et al*., 2014).

1.C Mitochondrial function

Maintenance of functional mtDNA is required for mitochondria's well-known role in cellular respiration and ATP production through OXPHOS. However, mitochondria play several additional critical roles in the cell; one of these additional functions is in iron-sulfur cluster biosynthesis. Budding yeast, for example, can survive under fermentation conditions without mtDNA but mitochondria are still required for iron-sulfur cluster biosynthesis. Iron-sulfur cluster biosynthesis is essential to almost all forms of life because iron-sulfur clusters are present in more than 200 different enzymes and are conserved in both prokaryotic and eukaryotic organisms (Bandyopadhyay *et al*., 2008). Defects in iron-sulfur cluster biosynthesis are linked to nuclear genome instability (Veatch *et al*., 2009). In addition, mitochondria also have functions in phospholipid biosynthesis, metabolite exchange, and programmed cell death (Figure 1.1). These functions of mitochondria are responsible for maintaining proper cellular function and organismal fitness. Defects in mitochondrial function are associated with many neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, and Parkinson's disease because neurons are highly dependent on mitochondrial function in ATP

production and Ca^{2+} buffering (Johri *et al*., 2012). Many studies have shown that mitochondrial functions are affected by mitochondrial shape and distribution and the integrity of mtDNA. Therefore, understanding the mechanisms that affect mitochondrial shape and distribution will contribute to knowledge about mitochondrial function and diseases that are associated with defects in mitochondrial shape and distribution.

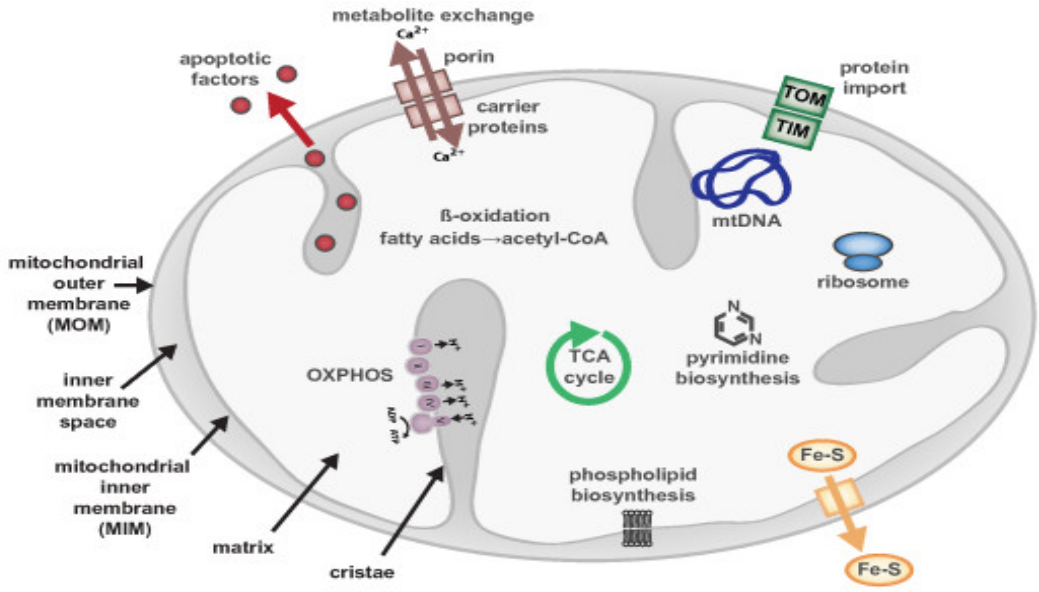


Figure 1.1: Mitochondria and mitochondrial functions. Mitochondria consist of inner and outer mitochondrial membranes with an intermembrane space enclosed between the inner and outer mitochondrial membrane and the matrix space enclosed by the inner membrane. Mitochondria play several critical roles including production of ATP via oxidative phosphorylation, phospholipid biosynthesis, metabolite exchange/buffering, β-oxidation of fatty acids, iron-sulfur cluster biogenesis, pyrimidine biosynthesis, and programmed cell death. TCA tricarboxylic acid. Adapted from Lackner, *BMC Biology*, 2014.

1.D Mitochondrial shape and distribution

In most eukaryotic cell types, mitochondria form highly dynamic, interconnected tubular networks that extend throughout cells (Figure 1.2). The shape and distribution of the mitochondrial network is critical for the proper function of mitochondria. Proper mitochondrial morphology is determined by four conserved activities: fusion, fission, motility and tethering

(Lackner *et al*., 2014). Fusion and fission are important for maintaining the health and dynamics of the mitochondrial network by altering the connectivity of mitochondrial tubules. Motility and tethering are necessary for the positioning and inheritance of mitochondria through trafficking and retention of mitochondria. There has been great progress made in understanding the molecular mechanisms of fusion and fission. However, the molecular mechanisms of motility and tethering and how these activities are regulated to position mitochondria are still poorly understood.

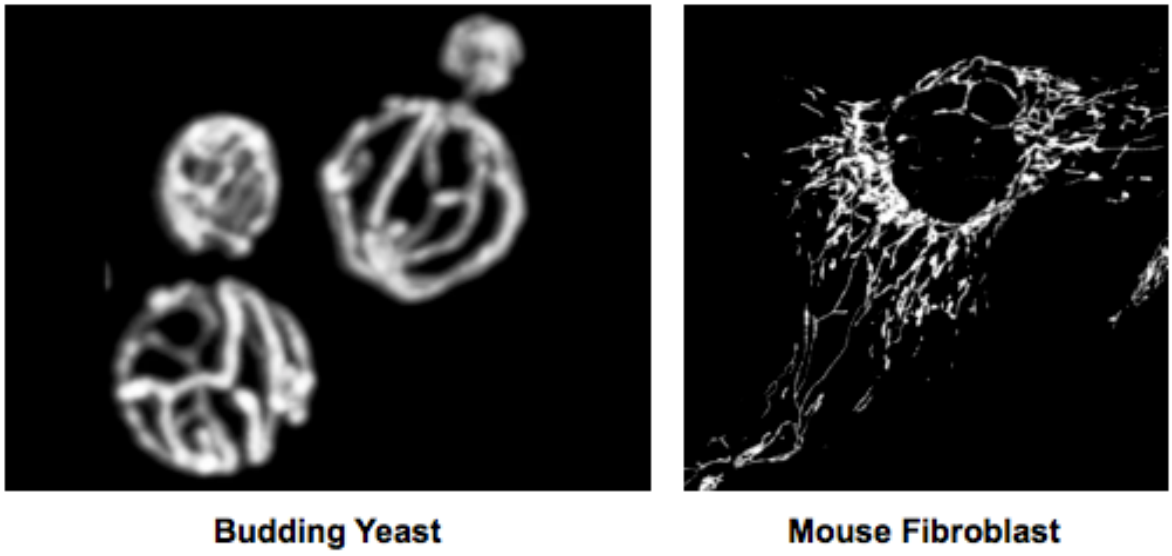


Figure 1.2: Mitochondrial distribution. In most eukaryotic cells, mitochondria form interconnected tubules that are distributed all throughout the cells. Budding yeast and mouse fibroblast are two representative eukaryotic cells. Adapted from Lackner, *BMC Biology* 2014 and Chen *et al*., 2013

1.E Mitochondrial positioning

Proper mitochondrial positioning, which involves the concerted mechanisms of transport and tethering, is critical to meet the specific needs of cells (van Bergeijk *et al*., 2016). Although active mitochondrial positioning is evidenced in various cell types such as neurons, oocytes of *Drosophila melanogaster*, stem cells, and yeast cells, the mechanism of transporting and

tethering are still poorly understood. Specifically, during asymmetric cell division, positioning ensures mother cells retain and daughter cells inherit the essential mitochondrial compartment, and likely plays a role in the asymmetric inheritance of new, higher functioning mitochondria by daughter cells (Westermann *et al*., 2014, McFaline-Figueroa *et al*., 2011). Defects in mitochondrial positioning are associated with aging, cancer and neurodegenerative diseases (McFaline-Figueroa *et al*., 2011, Mishra *et a*l., 2014, Nunnari *et al*., 2012).

1.E.1 Mitochondrial positioning in neurons

In neurons, mitochondria are transported down the axon and anchored near the synaptic terminals to serve as local providers of energy and calcium buffering (Ma *et al*., 2009, Chen *et al*., 2013). Mitochondrial distribution at the synaptic terminal is highly dynamic, which accommodates rapid changes in neuronal activity. At any given time, roughly one-third of neuronal mitochondria are mobile, while two-thirds remain stationary (Kraft *et al*., 2018). However, the ratio between motile and stationary mitochondria is not fixed and varies in response to changes in synaptic physiology. Each individual mitochondrion can rapidly switch between stationary and mobile states, altering mitochondrial distribution. Compared to other cells or even the cell body of a neuron, mitochondria appear more fragmented than interconnected in axons to facilitate their transport to and from the axon terminals. The depletion of a mitochondrial fission protein, dynamin-related protein 1 (Drp1), leads to a decrease in mitochondrial mass in axons and greatly impaired mitochondrial movements (Berthet *et al*., 2014).

In neurons, mitochondria are mobile, they are linked to the kinesin motor, KIF5, through an interaction with the mitochondrial outer membrane GTPase, Miro, and kinesin-binding

protein 1 and 2, Trak1 and 2 (Kang *et al*., 2008). However, in the presence of calcium, KIF5 binds to syntaphilin (SNPH), a mitochondrial tethering protein that anchors mitochondria to the microtubule cytoskeleton in neurons, and mitochondria become immobilized (Figure 1.3). Disrupting mitochondrial distribution at the synaptic terminal can lead to impairment of synaptic transmission. The mechanism of how the tethering complex is assembled and regulated and how a mitochondrion switches from an immobile to mobile state and vice versa are outstanding questions.

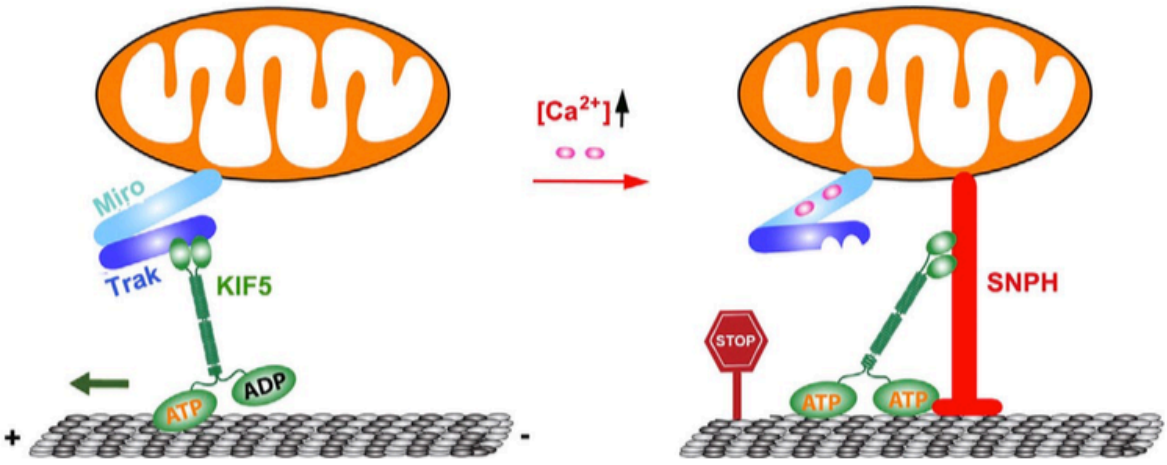


Figure 1.3: Activity-dependent tethering of mitochondria in axons. In the mobile state, mitochondria are linked to the kinesin motor, KIF5, through an interaction with the mitochondrial outer membrane GTPase, Miro, and kinesin-binding protein 1 and 2, Trak1 and 2. In response to elevated calcium (Ca2+), syntaphilin (SNPH) is recruited to mitochondria and functions to stall mitochondria. Adaptive from Chen *et al*., *Journal of Cell Biology*, 2013

1.E.2 Mitochondrial positioning in the oocytes of *Drosophila melanogaster*

In *Drosophila melanogaster*, mitochondria are passively transported to the posterior of the oocyte by cytoplasmic streaming and are retained there by the Long Oskar protein. This placement ensures the proper uptake of mitochondria and mtDNA by primordial germ cells (PGCs) (Hurd *et al*., 2016). The anchoring of mitochondria to the posterior site by Long Oskar requires the actin cytoskeleton (Figure 1.4). Disrupting the actin cytoskeleton causes

mitochondria to disassociate from the posterior of the oocyte. Additionally, in oocytes without Long Oskar, the number of mitochondrial genomes inherited by the PGCs is dramatically reduced, highlighting the essential role of Long Oskar and the actin cytoskeleton in mitochondrial inheritance. However, the mechanism of cytoskeletal-mediated mitochondrial localization and the role Long Oskar plays have yet to be determined. In addition, whether only functional mtDNA is retained at the posterior site and inherited by PGCs remains unknown.

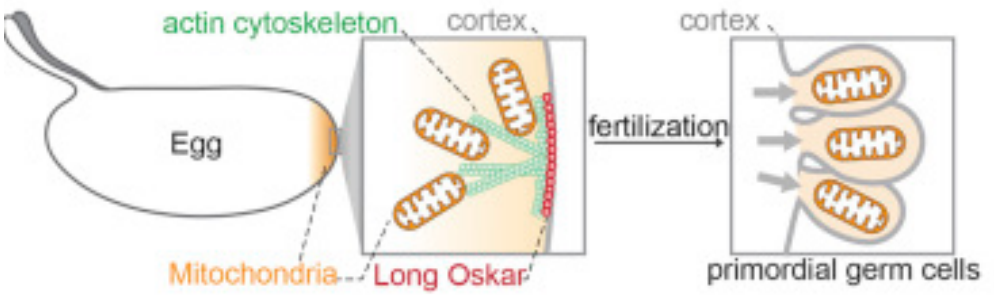


Figure 1.4: Long Oskar-dependent mitochondrial anchor in the *Drosophila* germline. Long Oskar anchors mitochondria to the posterior site through an unknown mechanism that requires the actin cytoskeleton. This anchorage is important for the inheritance mtDNA by the offspring. Adapted from Hurd *et al*., *Developmental Cell*, 2016

1.E.3 Mitochondrial positioning in asymmetrically dividing cells

Asymmetric cell division is a widespread process that occurs in both prokaryotes and eukaryotes. Proper organelle partitioning into the resulting cells is especially important in asymmetric cell division as the process gives rise to two distinct cells with different cytoplasmic components: one cell with older and more damaged cellular components and the other cell with younger and healthier components. Several organelles, such as mitochondria and ER cannot be generated *de novo*; therefore, these organelles must be differentially partitioned into the two resulting cells to ensure the critical amounts of organelles of the appropriate quality are segregated into the asymmetrically dividing cell.

Mitochondrial positioning prior to cell division is important for the asymmetry in stem cells undergoing cell division. In stem cells, asymmetric cell division plays a critical role in maintaining adult stem cell populations, while generating sufficient amounts of newly differentiated daughter cells to maintain tissue homeostasis. Defects in asymmetric cell division can result in excessive stem cell self-renewal and tumorigenesis (Toledano *et al*., 2009). Studies following mitochondrial partitioning in stem cell division showed prior to division, older mitochondria are spatially localized near the nucleus while the younger mitochondria are localized toward the cell cortex (Figure 1.5). Altering the localization of mitochondria decreased the asymmetric segregation of mitochondria into the resulting cells, which consequently decreased the stem-like characteristics in these daughter cells. Post cell division, daughter cells that receive a larger proportion of newly synthesized mitochondria maintain their stem-like characteristics, while cells that receive older mitochondria differentiate into somatic cells (Figure 1.5, Katajisto *et al*., 2015). While the positioning and differential partitioning of mitochondria play roles in maintaining stemness, the exact mechanism of how mitochondria are partitioned based on age into the daughter cell is poorly understood.

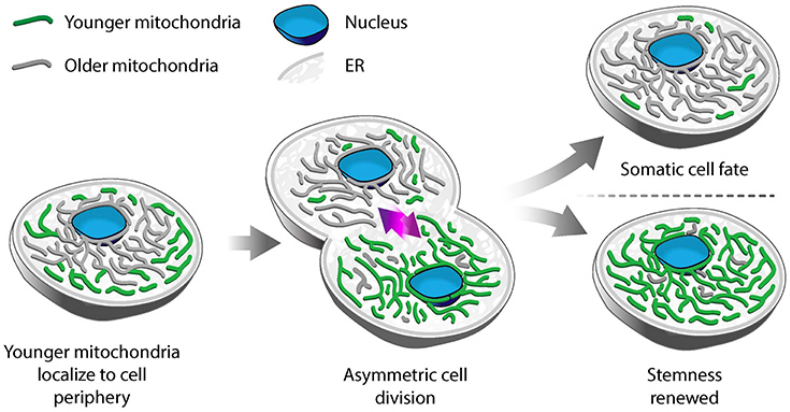


Figure 1.5: Asymmetric inheritance of mitochondria in stem cells. Mammalian stem cells divide asymmetrically. Cells that inherit more new mitochondria maintain their stemness, while cells that inherit more old mitochondria become somatic cells. Adapted Pernice *et al*., *frontiers in Cell Developmental Biology*, 2017.

Similar to the asymmetric characteristic of stem cells, budding yeast also divide asymmetrically. In budding yeast, positioning and inheritance of mitochondria has also been shown to play an essential role in asymmetric cell division. Scientists have long been using budding yeast in order to understand the mechanisms of cellular asymmetry in higher eukaryotes.

1.F *Saccharomyces cerevisiae* as a model organism

Saccharomyces cerevisiae (S. cerevisiae) is a powerful model organism for studying eukaryotic biology as many of the pathways and mechanisms in budding yeast are conserved in higher eukaryotes. Yeast cells are low maintenance unicellular eukaryotes that can be rapidly cultured in the lab at a relatively low cost. Yeast protein functions can be studied both *in vivo* and *in vitro* using a wide variety of biochemical and cell biological approaches. For studies *in vivo*, genes in yeast can easily be tagged with fluorescent proteins such as GFP and RFP to visualize the localization of the desired protein using fluorescence microscopy (Botstein *et al*., 2011). Yeast are also extremely amenable to genetic engineering techniques. We can study gene functions by introducing mutations at the endogenous locus to observe phenotypes in the absence of wildtype protein function (Botstein *et al*., 2011). For studies *in vitro,* proteins of interest can be overexpressed and purified for biochemical assays.

Work in yeast has provided a good foundation for our current understanding of organelle positioning and transport. Specifically, I utilize yeast to study organelle positioning in polarized cell types. During the cell cycle, yeast cells become polarized, and organelles are actively transported to the bud tip. As an asymmetrically dividing system, yeast have been used extensively to study inheritance and positioning mechanisms of various organelles. My project investigated the molecular mechanism and regulation of the inheritance of mitochondria during

the yeast cell cycle. By using yeast as a model organism, my work provides a basic understanding of mitochondrial positioning and inheritance that can be used to understand similar mechanisms in higher eukaryotic polarized cells, such as stem cells and neurons.

In addition, *S. cerevisiae* is also an ideal organism for studying mtDNA. The budding yeast mtDNA was completely sequenced in 1998 (Foury *et al.,* 1998, Freel *et al.,* 2015). Haploid *S. cerevisiae* is estimated to have ~50-200 copies of mtDNA. Interestingly, the organism is able to grow without functional mtDNA, in which case the cells are respiratory-deficient and form smaller colonies, termed petite colonies (Freel *et al.,* 2015). Since the cells are viable, this allows for the study of severe mitochondrial defects caused by mtDNA mutations. Moreover, there are a variety of well-characterized cell biological approaches to visualize mtDNA, such as tagging mtDNA-interacting proteins or by targeting fluorescent labels to mtDNA (Osman *et al.,* 2015). Therefore, yeast can be used to investigate the partitioning of mtDNA during the process of inheritance.

1.G The importance of mitochondrial and mtDNA positioning and inheritance in the aging of *S. cerevisiae*

In *S. cerevisiae*, aging can be defined in two ways: chronological aging and replicative aging. Chronological age is measured as the survival time of non-dividing yeast cells in stationary phase (chronological lifespan), while replicative age is measured as the number of daughter cells a mother cell can produce until she dies (replicative lifespan) (Longo *et al.*, 2012). On average, a mother cell can produce ~30 daughters before she dies. It is not known how asymmetric cell division contributes to the chronological lifespan of cells but studies have shown this mode of cell division produces cells with differing replicative lifespans. Interestingly,

despite the age of the mother cells, daughter cells are born young with full replicative potential. To ensure the rejuvenation of the daughters, budding yeast undergo asymmetric cell division where damaged cellular components are retained in the mother and healthy components are transported to the daughters. Damaged organelles, such as vacuoles with increased pH and mitochondria with decreased membrane potential, are retained in mothers, while the higher functioning form of these organelles are transported to and retained in the bud (Vevea *et al*., 2014). Studies have shown higher-functioning mitochondria, which are defined as mitochondria with higher membrane potential, higher reducing potential, and less reactive oxygen species, are inherited and positioned at the bud tip (McFalin-Figueroa *et al*., 2011, Pernice *et al*., 2016), and both the mitochondrial inheritance and retention mechanisms have been associated with aging. Additionally, recent studies suggest that mitochondrial inheritance mechanisms also play a role in the inheritance of mtDNA as mtDNA is often visualized in the tip of the mitochondrial tubule that first invades the bud (Osman *et al*., 2015). The contributions of mitochondrial inheritance mechanisms to mtDNA inheritance and the asymmetric aging of yeast are current areas of study.

1.H Mitochondrial inheritance in *S. cerevisiae*

During asymmetric cell division, mitochondria are transported to the bud after bud emergence and continue to move towards the bud tip, where they are retained throughout the cell cycle. While mitochondria must be inherited by the bud, some mitochondria must also be retained in the mother cell. This retention in the mother cell is facilitated by the mother specific tethering complex, MECA, and the protein Mfb1, both of which ensure proper distribution of mitochondria between the mother and the bud (Pernice *et al*., 2016, Ping *et al*., 2016).

Mitochondrial transport plays an important role in the asymmetric partitioning of mitochondria and the regulation of lifespan. There are two models to describe the mechanism that drives mitochondria into the bud and towards the bud tip, which is defined as anterograde movement. An older model suggests a motor-independent mode of mitochondrial movement into the bud during cell division. According to this model, mitochondria are linked to actin cables via the mitochore complex, which consists of Mdm10, Mdm12, and Mmm1, and the force generated by Arp2/3-dependent actin polymerization on the mitochondrial surface directs the movement of mitochondria to the bud (Fehrenbacher *et al.,* 2004, Boldogh *et al.,* 2006). Once mitochondria are transported into the bud, Mmr1 functions to tether mitochondria at the bud tip. However, this model has been challenged by studies that suggest Mmr1 plays a more direct role in transporting mitochondria, which will be discussed below. In addition, Mdm10, Mdm12, and Mmm1 are now known to be part of the ER-Mitochondria Encounter Structure (ERMES) that forms a mitochondria-ER contact site and is required to maintain mitochondrial morphology (Kornmann *et al*., 2010). The defects in mitochondrial inheritance observed in cells lacking these proteins is likely an indirect effect of severe defects in mitochondrial morphology. Further arguing against the model, new evidence suggests that actin polymerization on the mitochondrial surface plays a role in mitochondrial division rather than mitochondrial transport.

The second and more accepted model for mitochondrial transport suggests an active and motor-dependent movement of mitochondria. It has been shown Mmr1 and Ypt11 function to link mitochondria to the type V myosin motor Myo2, driving the transport of mitochondria to the bud (Figure 1.6, Itoh *et al*., 2004). Deleting either *MMR1* or *YPT11* leads to a delay in mitochondrial inheritance, and deleting both results in a severe mitochondrial inheritance defect in the W303 strain background and lethality in the BY4742 strain background (Frederick *et al*.,

2008, Itoh *et al*., 2004). The lethality of the mitochondrial inheritance defect can be rescued by artificially linking mitochondria to Myo2, suggesting Myo2 is essential for mitochondrial transport. Although Mmr1 and its partially redundant adaptor, Ypt11, are known mitochondrial adaptors, the molecular mechanisms and regulation of both adaptors are poorly understood. In addition to its role as a mitochondrial Myo2 adaptor, Mmr1 is also proposed to tether mitochondria to the bud tip via a physical interaction with cortical ER (Figure 1.6). If and how Mmr1 switches between its two functions are outstanding questions.

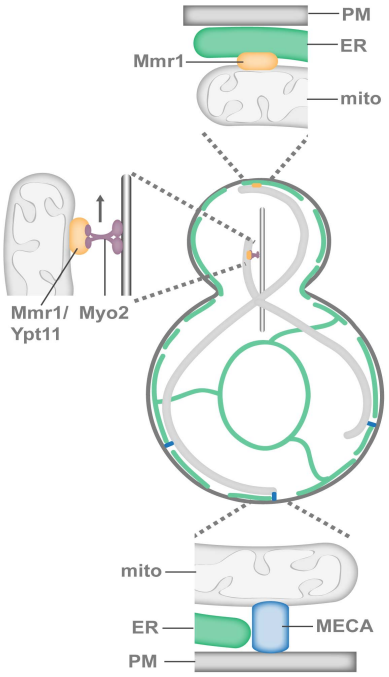


Figure 1.6: Mitochondrial inheritance and tethering in budding yeast. PM: plasma membrane, ER: endoplasmic reticulum, MECA: mitochondria-ER-cortex anchor

1.H.1 Motor-dependent mode of mitochondrial inheritance

Both Myo2 adaptors, Mmr1 and Ypt11, are important for motor-dependent mitochondrial inheritance. Overexpression of either *MMR1* or *YPT11* can rescue mitochondrial inheritance in W303 Δ*ypt11*Δ*mmr1* cells. Genetic evidence in BY4742 indicates that the Myo2 mutant, *myo2-573*, but not *myo2-338,* is lethal when combined with Δ*ypt11*. In contrast, *myo2-338*, but not *myo2-573*, is lethal when combined Δ*mmr1*. These data suggest the two adaptors interact with two distinct surfaces on Myo2 and function in parallel pathways for mitochondrial inheritance (Itoh *et al*., 2002, Itoh *et al*., 2004). Consistently, biochemical studies mapping the binding surface of various organelle specific adaptors on Myo2 showed the two mitochondrial adaptors, Mmr1 and Ypt11, have distinct Myo2 binding sites, further supporting the notion that these two adaptors function in parallel mitochondrial inheritance mechanisms (Eves *et al*., 2012).

1.H.2 Mitochondrial Myo2 receptor-related protein (Mmr1)

Mmr1 is a 491 amino acid protein with a molecular weight of 54.8 kDa. It contains a broadly defined mitochondria-binding region (61 – 355aa) and a well-studied Myo2-binding domain (378 – 441aa). Based on the Paircoil2 prediction program, amino acids 287-387 of Mmr1 form a coiled-coil domain (McDonnell *et al*., 2006, Figure 1.7). Mmr1 also contains two putative PEST sequences, which are thought to act as signaling peptides for degradation and are associated with short half-life proteins. In addition, Mmr1 contains 11 putative phosphorylation sites, one of which (S37) has been shown to be phosphorylated by Cdk1, and 2 putative ubiquitination sites (Holt *et al*., 2009).

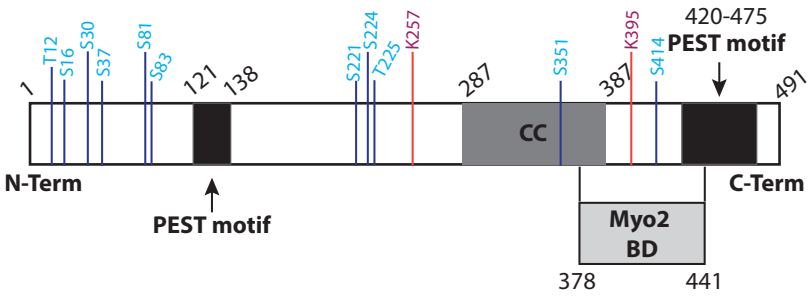


Figure 1.7: Schematic of Mmr1. Mmr1 consists of a predicted coiled-coil domain, a well-studied Myo2 binding domain, and two putative PEST motifs. The protein is also highly phosphorylated with 11 putative phosphorylation sites and two putative ubiquitination sites.

In budding yeast, Mmr1 has been shown to preferentially localize with mitochondria in the bud. Mmr1 is proposed to transport mitochondria to the bud and anchor mitochondria at the bud tip.

1.H.3 Mmr1 as a Myo2 adaptor for mitochondria

Mmr1 was identified during a screen for high copy suppressors of the temperature sensitive *myo2-573* mutant, which has a defect in mitochondrial inheritance and is lethal in

Δ*ypt11* cells at the non-permissive temperature (Itoh *et al*., 2004). In a different Myo2 mutant strain, where the Ypt11-Myo2 interaction is disrupted and mitochondrial inheritance is delayed, overexpression of *MMR1* rescues both the growth defect and mitochondrial distribution defect in cells, suggesting Mmr1 plays a positive role in mitochondrial inheritance. Furthermore, Δ*mmr1* cells exhibit a delay in trafficking mitochondria into the emerging bud and overexpression of *MMR1* causes mitochondria to accumulate in the bud.

Mmr1 co-immunoprecipitates with Myo2, but not with the *myo2-573* mutant suggesting Mmr1 and Myo2 form a complex in order to facilitate the transport of mitochondria and *myo2-573* disrupts the Mmr1-Myo2 interaction, hence disrupting the ability of Mmr1 to transport mitochondria (Itoh *et al*., 2004). A direct interaction between Mmr1 and Myo2 has been shown, and the binding regions on both proteins have been mapped. In addition, studies have shown Mmr1 localizes to mitochondria in the bud and can fractionate with mitochondria in subcellular fractionation assays (Itoh *et al*., 2004). Together, results from both genetic and biochemical approaches strongly suggest Mmr1 physically links mitochondria to Myo2 and define its role as an adaptor for mitochondrial inheritance.

1.H.4 Yeast Protein Two protein, Ypt11

Ypt11 is identified as Rab-type small GTPase based on its sequence conservation and function (Frei *et al*., 2006, Itoh *et al*., 2002). Rab-type small GTPases are a family of proteins important for the regulation of vesicle formation and the tethering and fusion of vesicles with their target membranes. Studies also demonstrate some Rab GTPases can interact with class V myosins. In melanocytes, Rab27a is important in facilitating melanosome transport by linking

melanophilin on the melanosome membrane to myosin-Va (Bahadoran *et al*., 2001). Similarly, Ypt11 interacts with class V myosin motors in yeast to facilitate organelle inheritance.

Ypt11 is composed of 417 amino acids, which is nearly two times longer than the typical length of Rabs. The additional length of Ypt11 results in Ypt11 containing unique sequence features that are either not found or are much longer than those in other Rabs. Unlike other Rabs, Ypt11 has an unusually long N-terminal extension, a C-terminal unstructured region, and an additional 83 amino acid insert that separates the P-loop and the switch I region of the conserved GTPase domain (Lewandowska *et al*., 2013). The C-terminus of Ypt11 contains a prenylation site necessary for membrane targeting. Removal of the prenylation site results in the cytosolic localization of Ypt11. The functional domain of Ypt11 remains largely unknown.

1.H.5 Ypt11 as a Myo2 adaptor for mitochondria

Similar to Mmr1, Ypt11 is a mitochondrial adaptor for Myo2. Deletion of *YPT11* results in a mitochondrial inheritance delay and overexpression of *YPT11* results in mitochondrial accumulation in the bud, suggesting Ypt11 has a role in the transport of mitochondria to the bud during cell division. Ypt11 was identified by coimmunoprecipitation (CoIP) and yeast two-hybrid (Y2H) assays to interact with Myo2. In CoIP assays, the tail domain of Myo2 interacts with Ypt11, and the *myo2-338* mutant fails to interact with Ypt11 as assessed by the Y2H assay. Consistently, in vivo *YPT11* overexpression in *myo2-338* cells failed to accumulate mitochondria in the bud. These data suggest Ypt11 facilitates mitochondrial inheritance and the interaction between Ypt11 and Myo2 is important for this function.

Unlike Mmr1, the function of Ypt11 in organelle inheritance is not specific for mitochondria. Studies have shown that Ypt11 also functions in the transport of cER and late

Golgi membranes. Plasmid expressed GFP-Ypt11 localizes to the growing bud. However, in *myo2-338* and *ypt11* mutant cells, where Ypt11 can no longer interact with Myo2, the localization of Ypt11 becomes dispersed and shifts to the cER or perinuclear ER. In addition, the overexpression of *YPT11* also results in the localization of Ypt11 to the cER. These data suggest Ypt11 requires interaction with Myo2 for bud localization. Given the function of Ypt11 in the inheritance of multiple organelles, the role of Ypt11 in mitochondrial inheritance is more complicated to dissect.

1.H.6 Retrograde transport

In the case of mitochondrial inheritance, anterograde movement drives the movement of mitochondria from mothers to buds. Time-lapse imaging reveals mitochondria also engage in retrograde movement, mitochondrial movement from buds to mothers (Fehrenbacher *et al*., 2004). This retrograde mitochondrial movement is associated with actin cables undergoing retrograde flow. Formins are actin nucleation proteins that polymerize actin at the plus ends of actin filaments near the bud tip, providing a track for Myo2, a plus end directed motor, to move mitochondria toward the bud neck or the bud tip of the cell. The continuous polymerization of actin cables by formins at the bud tip generates a retrograde actin flow (Huckaba *et al*., 2004). The anterograde mitochondrial movement must overcome retrograde actin flow in order to transport mitochondria into the bud (Fehrenbacher *et al*., 2004).

1.I Mitochondrial tethering in budding yeast and during cell division

In addition to the active transport of mitochondria, mitochondrial anchorage is also important in the proper partitioning of the mitochondrial network. During yeast asymmetric cell

division, mitochondria are transported to the bud and anchored at the bud tip as well as the mother cortex to ensure mother cells retain and daughter cells inherit the essential mitochondrial compartment. There are three tethers involved in mitochondrial positioning in budding yeast: one bud specific tether - Mmr1, and two mother specific tethers – the mitochondria-ER-cortex anchor, MECA, and mitochondrial F-box-containing protein, Mfb1, (Figure 1.8, Ping *et al*., 2016, Pernice *et al*., 2016, Pernice *et al*., 2017, McFaline-Figueroa *et al*., 2011).

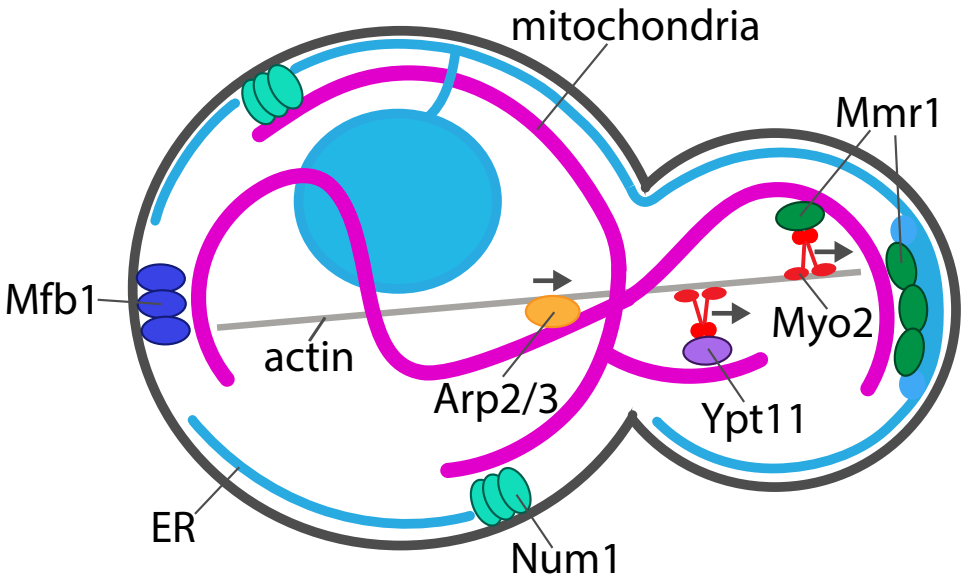


Figure 1.8: Mitochondrial positioning mechanisms. Arp2/3 and Mmr1/Ypt11 are active mitochondrial transport mechanisms, while Mmr1, Num1 and Mfb1 are mitochondrial tethering mechanisms.

In the bud, Mmr1 has been suggested to tether mitochondria to the bud tip post transport. Long-term live cell imaging shows Mmr1 and mitochondria colocalize at the bud tip for long durations. Deletion of *MMR1* results in mitochondria no longer accumulating at the bud tip, and mitochondrial retrograde transport, i.e. movement toward the mother cell, increases in Δ*mmr1* cells. These data suggest Mmr1 is responsible for maintaining mitochondria at the bud tip. Super-resolution structured illumination imaging shows that Mmr1 localizes to mitochondria at

the bud tip proximal to cER sheets. In addition, subcellular fractionation indicates a small fraction of Mmr1 is associated with the ER. Together, these observations suggest the anchorage interface for mitochondria at the bud tip is the cER (Swayne *et al*., 2011). Thus, Mmr1 plays an important role in mitochondrial inheritance by facilitating mitochondrial transport to the bud and mitochondrial positioning at the bud tip. However, the mechanism underlying the switch in Mmr1 function from transport to tethering and its regulation are unknown.

MECA anchors mitochondria to the plasma membrane (PM) in the mother cell. It is a multi-subunit complex composed of the core component Num1 and accessory protein Mdm36. Deletion of Num1 abolishes the cortical anchorage of mitochondria in mother cells. This results in the accumulation of mitochondria in the bud, as more mitochondria are inherited due to the lack of retention in the mother. Conversely, mitochondria accumulate in the mother in cells that lack Mmr1 or when Num1 is overexpressed (Westermann *et al*., 2014, Klecker *et al*., 2014). In addition, there is a positive genetic interaction between *NUM1* and *MMR1*, suggesting the two proteins have antagonistic functions. Deletion of both proteins rescues the unequal distribution of mitochondria between mother and bud and restores proper mitochondrial partitioning (Westermann *et al*., 2014, Klecker *et al*., 2014). Thus, these two proteins work together to ensure proper mitochondria distribution during cell division.

In addition to the MECA complex, mitochondrial F-box protein, Mfb1, has been shown to be enriched in the distal end of the mother cell and is required for anchoring mitochondria at that site. Mitochondrial accumulation at the distal end of the mother cells and Mfb1 localization are unaltered in Δ*num1* cells, in which most mitochondria-cortex anchors are abolished, suggesting Mfb1-dependent mitochondrial anchorage is independent of Num1. In addition, there is a positive genetic interaction between *MFB1* and *MMR1*, suggesting the two proteins have

antagonistic functions (Pernice *et al*., 2015). Deletion of *MFB1* resulted in 86% depletion of mitochondria in the distal end of the mother suggesting the accumulation of mitochondria at the mother cell tip largely depends on Mfb1 (Pernice *et al*., 2015).

These three known mitochondrial tether proteins work together to distribute and position the mitochondrial network throughout the budding yeast cell and during cell division. More specifically some of these tethering proteins are important to the asymmetric inheritance of higher functioning mitochondria by daughter cells.

1.J The role of mitochondrial inheritance and positioning in maintaining asymmetric partitioning of mitochondria

Mitochondrial function is not homogenous across the cell; instead higher functioning mitochondria are retained at regions on opposite ends of the cells. The positioning of higher functioning mitochondria is regulated by retention mechanisms. Mmr1 and Mfb1 have specifically been shown to anchor higher functioning mitochondria at the bud tip and the distal end of the mother, respectively (Figure 1.9). Measurements of mitochondrial ROS and redox state indicate that mitochondria anchored at the bud tip have less ROS and are more reducing compare to mother cell mitochondria (Pernice *et al*., 2016, McFaline-Figueroa *et al*., 2011). In addition, mitochondria in the mother cell have been shown to be physically separated from the mitochondria in the bud, creating two distinct networks of mitochondria (McFaline-Figueroa *et al*., 2011).

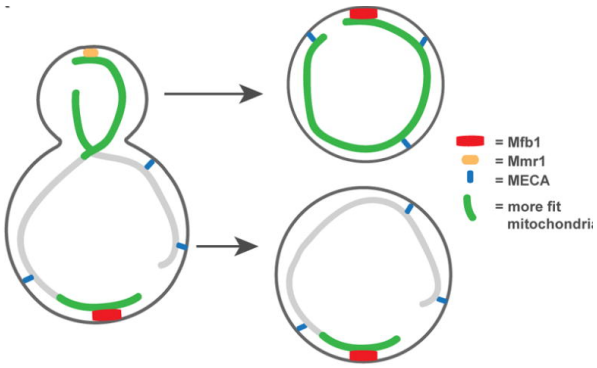


Figure 1.9: Asymmetric inheritance of mitochondria in yeast. During yeast cell division, daughter cells inherit higher functioning mitochondria. Mmr1 and Mfb1 specifically anchor higher functioning mitochondria at the bud tip and the distal end of the mother. Adapted from Kraft and Lackner, *Biochemical and Biophysical Research Communications*, 2018.

Deletion of *MMR1* affects the replicative lifespan of yeast resulting in one population of cells with a longer life span and another with a shorter life span compared to wildtype (McFaline-Figueroa *et al*., 2011). This suggests that Mmr1 plays an important role in affecting cellular asymmetry and specifically the replicative lifespan newborn cells. Since Mmr1 has been shown to function in both the active transport and retention of mitochondria, it is hard to differentiate which of the functions are important for regulating mitochondrial fitness and age asymmetry.

Deletion of *MFB1* in wildtype cells significantly reduces the localization of higher functioning mitochondria at the maternal distal end and decreases the replicative lifespan of cells. Deletion of *MFB1* in Δ*mmr1* cells rescues the aging defects of Δ*mmr1* cells, further suggesting the two tethering proteins have antagonistic functions. On the other hand, Num1, the other mitochondria anchoring protein in the mother, is suggested to be insufficient for the anchorage of higher functioning mitochondria in the mother (Pernice *et al*., 2015).

Together, mitochondrial trafficking and tethering ensure proper mitochondrial distribution and likely play roles in the asymmetric inheritance of new, higher functioning

mitochondria by daughter cells. Mitochondrial function is assessed by membrane potential, reducing potential, ATP production, and reactive oxygen species, which are affected by the function of the OXPHOS pathway and mtDNA. There are many outstanding questions regarding the effects of mtDNA and its inheritance on the replicative aging of cells: 1) Does mtDNA affect mitochondrial inheritance and age asymmetry? 2) If so, how does mtDNA affect mitochondrial inheritance? 3) Do proteins involved in either transport or tethering also affect mtDNA inheritance?

1.K The distribution of mitochondrial DNA

One of the key components of mitochondria, mtDNA, is packaged into nucleoids and many nucleoids are evenly distributed throughout the mitochondrial network (Figure 1.10. Studies in yeast examining the correlation between mitochondrial network length and the number of nucleoids in each cell showed nucleoids are evenly distributed with a consistently observed distance of ~800 nm (Osman *et al*., 2015). This even distribution of the mitochondrial genome is important for the distribution of OXPHOS complexes throughout the mitochondrial network. Hence, examining the mechanisms of nucleoid distribution throughout the cell is important.

Interestingly, studies also indicate that the physical movement of nucleoids within the mitochondrial network is limited, suggesting the distribution of nucleoids relies on the dynamics

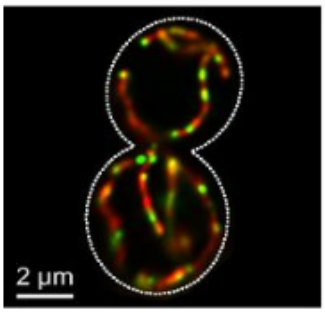


Figure 1.10: Nucleoid distribution. In budding yeast, nucleoids are well distributed throughout the mitochondrial network. Adapted from Osman *et al*., *Proceedings of the National Academy of Sciences*, 2015.

of the mitochondrial network. Consistently, studies show nucleoid distribution depends on mitochondrial division. In yeast as well as mammalian cells, the majority of mtDNA replication is observed in close proximity to mitochondria-ER contact sites, which also mark mitochondrial division sites. When DNA replication occurs at the site of mitochondrial division, 60% of the events result in a nucleoid being partitioned into each of the resulting tips (Murley *et al*., 2013, Lewis *et al*., 2016). This suggests that ER-associated mitochondrial division ensures that the majority of mitochondrial tips contain a nucleoid. In yeast, 69% of the tips after division have been shown to contain mtDNA <500 nm away from the tip of a mitochondrial tubule (Osman *et al*., 2015). These tips can be transported over long distances, where they can fuse with other mitochondrial tubules in order to distribute mtDNA throughout the mitochondrial network.

1.L mtDNA inheritance in budding yeast

The active partitioning of mitochondria requires the motor protein Myo2 and Myo2 adaptors, Mmr1 or Ypt11, to transport the organelle on actin cables, as discussed previously. On the other hand, the partitioning of mtDNA is less well understood. There are two possible non-mutually exclusive models for mtDNA partitioning into the daughter cells: the 'spacing-model' and the 'active sorting' model. The 'spacing-model' states a uniform mtDNA distribution throughout the mitochondrial network is essential for faithful partitioning of mitochondria into daughter cells (Jajoo *et al*., 2016). The 'active sorting' model states the active transport of mitochondria via molecular motors is essential for the movement of mtDNA to the daughter cells and facilitates the faithful partitioning of mitochondria and mtDNA.

Data obtained from *S. pombe,* where cells divide symmetrically, support the 'spacing model', in which faithful segregation of mtDNA relies on even spacing between nucleoids in the

mitochondrial network and the well distributed positioning of mitochondria throughout the cytoplasm (Jajoo *et al*., 2016). Disruption of mitochondrial distribution prior to cytokinesis results in partitioning of unequal amounts of mitochondrial volume and mtDNA into daughter cells. This study showed the even distribution of mtDNA throughout the mitochondrial network is important for the partitioning of mtDNA and partitioning of mtDNA is dependent on the partitioning the mitochondrial compartment. Yet, the molecular mechanisms underlying the even spacing of nucleoids are not known.

In asymmetrically dividing cells like *S. cerevisiae*, being able to faithfully partition mtDNA, specifically functional mtDNA, is essential in maintaining respiratory competent mitochondria. Studies have shown that mtDNA has limited mobility within the mitochondrial network, yet mtDNA is often localized to the mitochondrial tip that invades the bud. It is observed that in 90% of the cases in which a mitochondrial tip invades a bud, mtDNA is localized to the invading tip, suggesting mtDNA tip localization is important for the inheritance of mtDNA in the daughter cell (Osman *et al*., 2015). Previous studies showed mtDNA tip localization might result from mitochondrial division. However, in cells lacking both *DNM1*, a dynamin-related GTPase required for mitochondrial fission, and *FZO1*, a gene required for mitochondrial outer membrane fusion, mtDNA distribution and inheritance are unaffected suggesting neither fission or fusion is essential for faithful distribution and the inheritance of mtDNA (Osman *et al*., 2015). Further investigation demonstrated an alternative mode of tip generation that is independent of fission and fusion; the majority of such tips are closely associated with mtDNA. Disrupting the actin cytoskeleton in Δ*dnm1*Δ*fzo1* cells disrupts fission-independent tip generation (Osman *et al*., 2015). Together, these data suggest a fission-

independent and actin-dependent mechanism is important for generating mitochondrial tips that contain mtDNA in Δ*dnm1*Δ*fzo1* cells.

Many unanswered questions are actively being investigated in the field in order to understand how the inheritance of mtDNA is coupled with mitochondrial inheritance during cell division: 1) What are the molecular mechanisms that ensure mtDNA is faithfully inherited with the rest of the mitochondrial compartment? and 2) Is mtDNA physically linked to the machinery that drives mitochondrial motility?

1.M Summary

While many interesting observations about mitochondrial inheritance have been made, the mechanism behind the inheritance of higher functioning mitochondria and functional mtDNA remains largely unknown. My thesis research examines the molecular mechanism of Mmr1-dependent mitochondrial inheritance, which has also been implicated to play an important role in the inheritance of higher functioning mitochondria. Moreover, my work reveals a potential novel role of Mmr1 in maintaining mtDNA integrity, which opens many avenues for future research.

Chapter 2

Direct membrane binding and self-interaction contribute to Mmr1 function in mitochondrial inheritance

The data in this chapter were published in Molecular Biology of the Cell. Chen, W, HA Ping, and LL Lackner. "Direct Membrane Binding and Self-Interaction Contribute to Mmr1 Function in Mitochondrial Inheritance." *Mol Biol Cell* 29, no. 19 (2018): 2346–57. PMID: 30044712 (https://doi.org/10.1091/mbc.E18-02-0122)
Most of the data were generated by WeiTing Chen, with the help from the other contributing authors.

2.A Introduction

Mitochondrial positioning is an active and regulated process that couples the distribution of the organelle with the needs of the cell. The position of mitochondria is determined in part by the activities of mitochondrial transport and anchoring (Labbe *et al*., 2014; Lackner, 2014). Coordinated regulation of these activities ensures mitochondria are trafficked to and dynamically maintained at specific cellular locations. For example, mitochondria are positioned in specific regions of activated immune cells and axons to serve as local providers of energy and calcium buffering (Chada and Hollenbeck, 2004; Quintana *et al*., 2007; Schwarz, 2013; Lin and Sheng, 2015). The positioning of mitochondria at the oocyte posterior in *Drosophila* is required for the efficient incorporation of mitochondria into primordial germ cells (Hurd *et al*., 2016). In addition, in asymmetrically dividing cell types such as yeast and stem-like cells, mitochondrial positioning pathways likely influence the asymmetric age/function-dependent inheritance of mitochondria, which affects the fate of each daughter (McFaline-Figueroa *et al*., 2011; Katajisto *et al*., 2015; Pernice *et al*., 2016; Pernice *et al*., 2017; Kraft and Lackner, 2018). While players in mitochondrial positioning pathways have been identified, a deeper understanding of the molecular mechanism as well as the spatial, temporal, and contextual regulation of these proteins is required to understand how mitochondria are positioned at the right place and time to meet cellular needs.

In yeast, the antagonistic functions of bud and mother cell positioning mechanisms govern the partitioning of mitochondria between the mother and daughter. Mitochondrial transport to the bud begins early in the cell cycle and is dependent on Myo2, a type V myosin that drives actin-based transport of mitochondria to the bud (Simon *et al*., 1997; Itoh *et al*., 2002; Altmann *et al*.,

2008; Fortsch *et al*., 2011). Mmr1 and Ypt11 function as adaptors that link mitochondria to Myo2, and either Mmr1 or Ypt11 are required for Myo2-dependent transport of mitochondria to buds (Itoh *et al*., 2002; Boldogh *et al*., 2004; Itoh *et al*., 2004; Frederick *et al*., 2008; Eves *et al*., 2012; Chernyakov *et al*., 2013; Lewandowska *et al*., 2013). Mmr1 is also proposed to function in the retention of mitochondria in buds by physically tethering mitochondria to cortical ER sheets at the bud tip (Swayne *et al*., 2011). The movement into and anchoring of mitochondria in buds are counterbalanced by two mitochondrial anchors that function to retain mitochondria in mother cells, the mitochondria-ER-cortex anchor (MECA) and Mfb1 (Cerveny *et al*., 2007; Klecker *et al*., 2013; Lackner *et al*., 2013; Pernice *et al*., 2016). How the localization and activity of these proteins are regulated in space and time to govern the distribution and inheritance of the mitochondrial network over the course of the cell cycle are poorly understood.

Mmr1 must interact with both mitochondria and Myo2 to function in mitochondrial positioning. A Myo2-binding domain within Mmr1 has been characterized and shown to be sufficient for interaction with the motor (Itoh *et al*., 2004; Eves *et al*., 2012). A mitochondrial binding region within Mmr1 has also been described (Itoh *et al*., 2004). However, the molecular basis of the interaction between Mmr1 and mitochondria is undefined. In addition, the contributions of Mmr1's predicted coiled-coil domain to overall Mmr1 function are not clear (Itoh *et al*., 2004). Here, we use a structure-function analysis of Mmr1 to gain insight into the functional contributions of various Mmr1 domains. We identified a membrane binding domain in Mmr1 that is required for the interaction with mitochondria and Mmr1-mediated mitochondrial inheritance. In addition, our studies indicate the coiled-coil domain of Mmr1 mediates an Mmr1-Mmr1 interaction, which facilitates the polarized localization of the protein and, consequently,

impacts Mmr1 function. We predict that the activities of direct membrane binding and self-interaction are regulated to spatially and temporally control Mmr1 function in the cell.

2.B Mmr1 interacts directly with phospholipid membranes

Mmr1 is a soluble protein that interacts peripherally with mitochondria (Itoh *et al*., 2004). However, the molecular basis for the Mmr1-mitochondria interaction is unknown. We have shown that Num1, the core protein component of the mitochondrial tether MECA (Lackner *et al*., 2013), interacts directly with the mitochondrial membrane (Ping *et al*., 2016). To test if Mmr1 is also able to directly interact with phospholipid membranes, we examined the membrane binding properties of Mmr1 in vitro. Recombinant Mmr1 was purified from *Escherichia coli*, and its ability to associate with liposomes that mimic the composition of the mitochondrial outer membrane was examined using liposome floatation assays. Specifically, we used individual phospholipids to make chemically defined liposomes that mimic the composition of the mitochondrial outer membrane (outer membrane composition; OMC) and varied the concentration of cardiolipin (CL) present in these liposomes (0%, 6%, and 17%). CL, a mitochondria-specific phospholipid, is reported to be present at 6% in the mitochondrial outer membrane and at 17% at contact sites between the mitochondrial outer and inner membranes (Simbeni *et al*., 1991; Zinser and Daum, 1995). In floatation assays, recombinant Mmr1 associated with OMC liposomes containing 6% and 17% CL, but not with OMC liposomes that lack CL (Figure 2.1A).

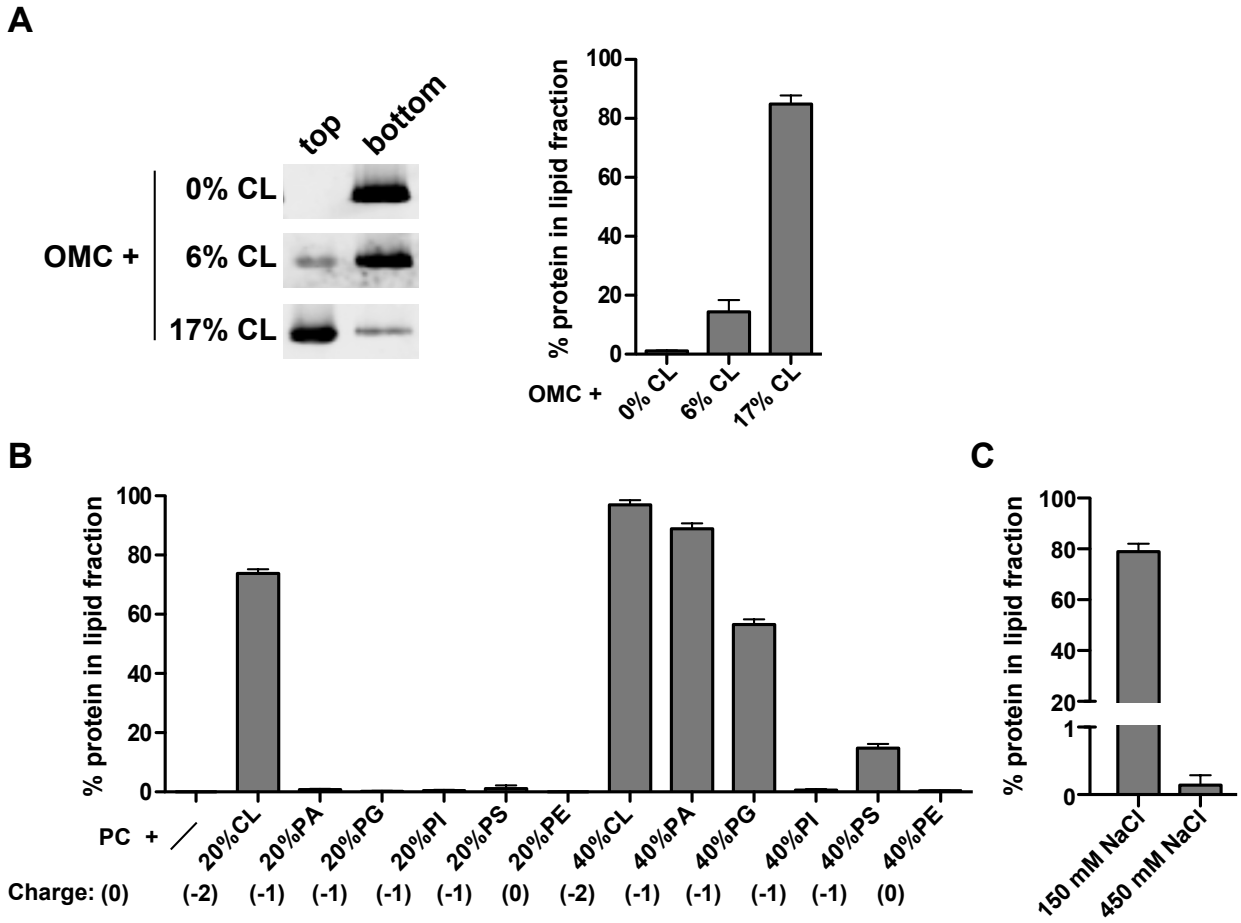


Figure 2.1: Mmr1 interacts directly with phospholipid membranes. (A) Purified Mmr1 (5 μM) was incubated with OMC liposomes containing 0, 6, and 17% CL, as indicated. The association of protein with liposomes was assessed by its ability to float with liposomes, as indicated by the amount of protein in the top fraction of the gradient. Equivalent amounts of the top and bottom fractions of the flotation gradients were subjected to SDS–PAGE and Western blot analysis (left panel). The percentage of protein found in the top fraction is shown as the mean ± SEM; $n = 3$ independent experiments. (B) Purified Mmr1 (5 μM) was incubated with liposomes composed of PC and the indicated mol% of a second phospholipid, and the reactions were subjected to liposome flotation and analyzed as described in A. Data are shown as the mean ± SEM; $n = 3$ independent experiments. The net charge of the phospholipid headgroups is indicated below the graph in parentheses. (C) Purified Mmr1 (5 μM) was incubated with PC + 20% CL liposomes in the presence of 150 or 450 mM NaCl. The reactions were subjected to liposome flotation and analyzed as described in A. Data are shown as the mean ± SEM; $n = 3$ independent experiments.

To further examine the specificity of the Mmr1-phosholipid interaction, we assessed the ability of Mmr1 to bind liposomes composed of the neutral phospholipid phosphatidylcholine (PC) plus one of the following phospholipids: cardiolipin (CL), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), or

phosphatidylethanolamine (PE). When individual phospholipids were present at 20 mol%, Mmr1 only associated with CL-containing liposomes (Figure 2.1B). When increased to 40 mol%, PA, PG, and PS were also able to support the Mmr1-phospholipid interaction. Increasing the salt concentration in the liposome floatation assays disrupted the Mmr1-phospholipid interaction, indicating that the interaction is electrostatic (Figure 2.1C). Together, these data indicate that Mmr1 can interact directly with phospholipid membranes in vitro and exhibits a preference for liposomes containing CL.

2.C Mmr1(76-195) is necessary and sufficient for the interaction with mitochondria

Mmr1 lacks a predicted membrane-binding domain. To identify the membrane-binding domain within Mmr1, we expressed a series of Mmr1 truncations as yEGFP fusions from the endogenous *MMR1* locus and examined their localization relative to mitochondria (Figure 2.2A). Western blot analysis confirmed that the proteins were expressed with minimal degradation (Figure 2.2B). We based our truncations on the results of a previous study, which mapped the mitochondrial binding domain of Mmr1 to amino acids 61-355 (Itoh *et al*., 2004), and on the results of structure prediction programs and regions of conservation. Consistent with previous studies, we observed that full length Mmr1 co-localized with mitochondria and exhibited a punctate, bud-enriched localization (Figure 2.2A; (Itoh *et al*., 2004; Swayne *et al*., 2011; Eves *et al*., 2012). For the Mmr1 truncations, we found that Mmr1(61-195) co-localized with mitochondria, while the distribution of Mmr1(61-152) was shifted towards the cytosol. In addition, we found that Mmr1(76-195) co-localized with mitochondria but Mmr1(91-195) and Mmr1(76-152) were primarily cytosolic.

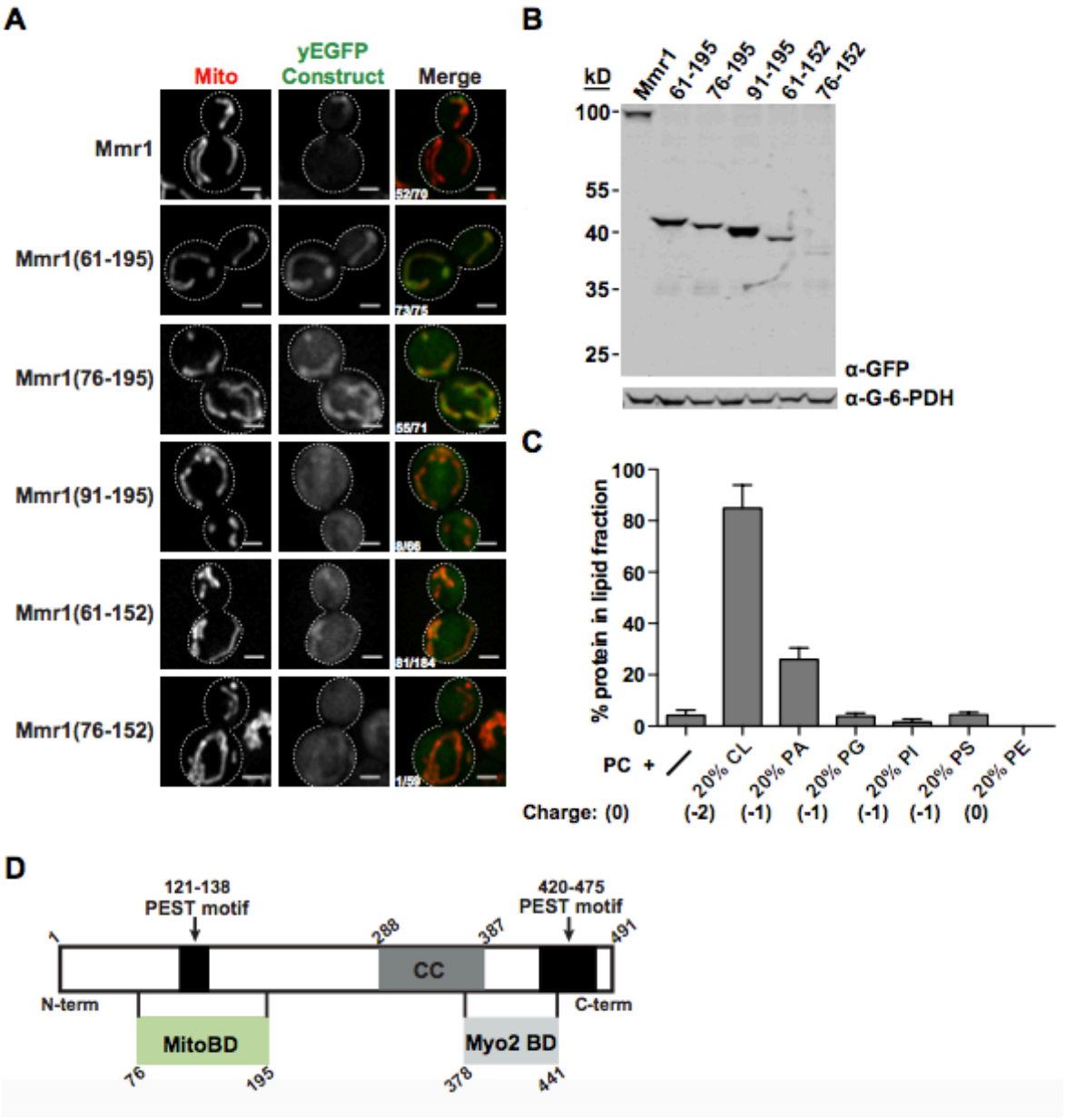


Figure 2.2: Amino acids 76–195 of Mmr1 are sufficient for the interaction with mitochondria. (A) Cells expressing mitoRED and the indicated Mmr1-yEGFP truncations were analyzed by fluorescence microscopy. Whole cell projections are shown. The cell cortex is outlined with a dashed white line. Scale bar, 2 μm. The number of cells in which the Mmr1-yEGFP truncation was observed to colocalize with mitochondria out of the total number of cells counted is shown in the bottom left corner of the merge image panel. (B) Whole cell extracts of strains expressing truncated forms of Mmr1-yEGFP, as indicated, were analyzed by SDS–PAGE and Western blot using anti-GFP to detect the Mmr1-yEGFP truncations and anti-G-6-PDH as a loading control. (C) Purified Mmr1(61–195)-GFP (5 μM) was incubated with liposomes composed of PC and the indicated mol% of a second phospholipid. The reactions were subjected to liposome flotation and analyzed as described in Figure 2.1A. Data are shown as the mean ± SEM; n = 3 independent experiments. The net charge of the phospholipid headgroups is indicated below the graph in parentheses. (D) Schematic of Mmr1. Myo2 BD, Myo2-binding domain; CC, coiled-coil; MitoBD, mitochondrial binding domain.

In contrast to the polarized localization of wildtype Mmr1, Mmr1(76-195) and the other mitochondrial-associated Mmr1 truncations appeared to be evenly distributed along mitochondria in the mother and bud (Figure 2.2A). These proteins lack the Myo2 binding domain, and their localization is consistent with the loss of Myo2-dependent bud polarized localization (Itoh *et al*., 2004; Eves *et al*., 2012). Together, these results indicate that Mmr1(76-195) is sufficient for the interaction with mitochondria.

We next asked whether the minimal mitochondrial binding domain, Mmr1(76-195), was sufficient to interact with phospholipid membranes in vitro. Recombinant Mmr1(76-195) could not be stably expressed in *E. coli*. However, we were able to express and purify recombinant Mmr1(61-195) and test its ability to associate with phospholipid membranes using liposome floatation assays. Similar to full length Mmr1, Mmr1(61-195) directly associated with liposomes and exhibited a preference for CL- and PA-containing phospholipid membranes (Figure 2.2C). Thus, our in vivo and in vitro studies indicate that amino acids 76-195 of Mmr1 compose the mitochondrial binding domain (mitoBD; Figure 2.2D).

We then examined whether our defined mitoBD was necessary for the Mmr1-mitochondria interaction in cells. We expressed Mmr1Δ76-195-yEGFP from the endogenous *MMR1* locus and examined the localization of the protein relative to mitochondria. We observed that Mmr1Δ76-195-yEGFP no longer co-localized with mitochondria (Figure 2.3A), consistent with the disruption of the Mmr1-mitochondria interaction. In addition, an enrichment of Mmr1Δ76-195-yEGFP in small buds was observed (Figure 2.3A and B), indicating that deletion of the mitochondrial binding domain did not disrupt the overall folding of Mmr1 and the protein was

still able to interact with Myo2. Western blot analysis confirmed that the protein was expressed with minimal degradation (Figure 2.4A). As discussed below, Mmr1Δ76-195 was also able to interact with itself in yeast two-hybrid assays (Figure 2.5B), providing further evidence that deletion of amino acids 76-195 specifically disrupts the interaction between Mmr1 and mitochondria.

We next sought to identify amino acids within the mitoBD that when mutated disrupt the Mmr1-mitochondria interaction in vivo and examine how these mutations affect the ability of the protein to bind phospholipid membranes in vitro. Given the affinity of Mmr1 for negatively charged lipids, we identified basic amino acids within the Mmr1 mitoBD that are conserved and reversed the charge of these amino acids (Figure 2.4B). Specifically, we constructed Mmr1 R80E R86E K95E K98E and will refer to this mutant as Mmr1^{4E}. When expressed as a yEGFP fusion in cells, Mmr1^{4E} no longer associated with mitochondria (Figure 2.3A and Figure 2.4A), indicating the mutations interfere with the Mmr1-mitochondria interaction. In addition, the protein was found to be enriched in small buds, indicating that the interaction between Mmr1^{4E} and Myo2 was not disrupted (Figure 2.3A and B). We then purified Mmr1^{4E} and tested its ability to interact directly with phospholipid membranes in vitro. In comparison to wildtype Mmr1, the association of Mmr1^{4E} with OMC+17% CL liposomes was dramatically reduced (Figure 2.3C). Thus, the inability of Mmr1^{4E} to associate with mitochondria in cells correlates with a defect in phospholipid membrane binding in vitro. Together, our data suggest that the Mmr1 mitoBD mediates a direct interaction between Mmr1 and the mitochondrial membrane.

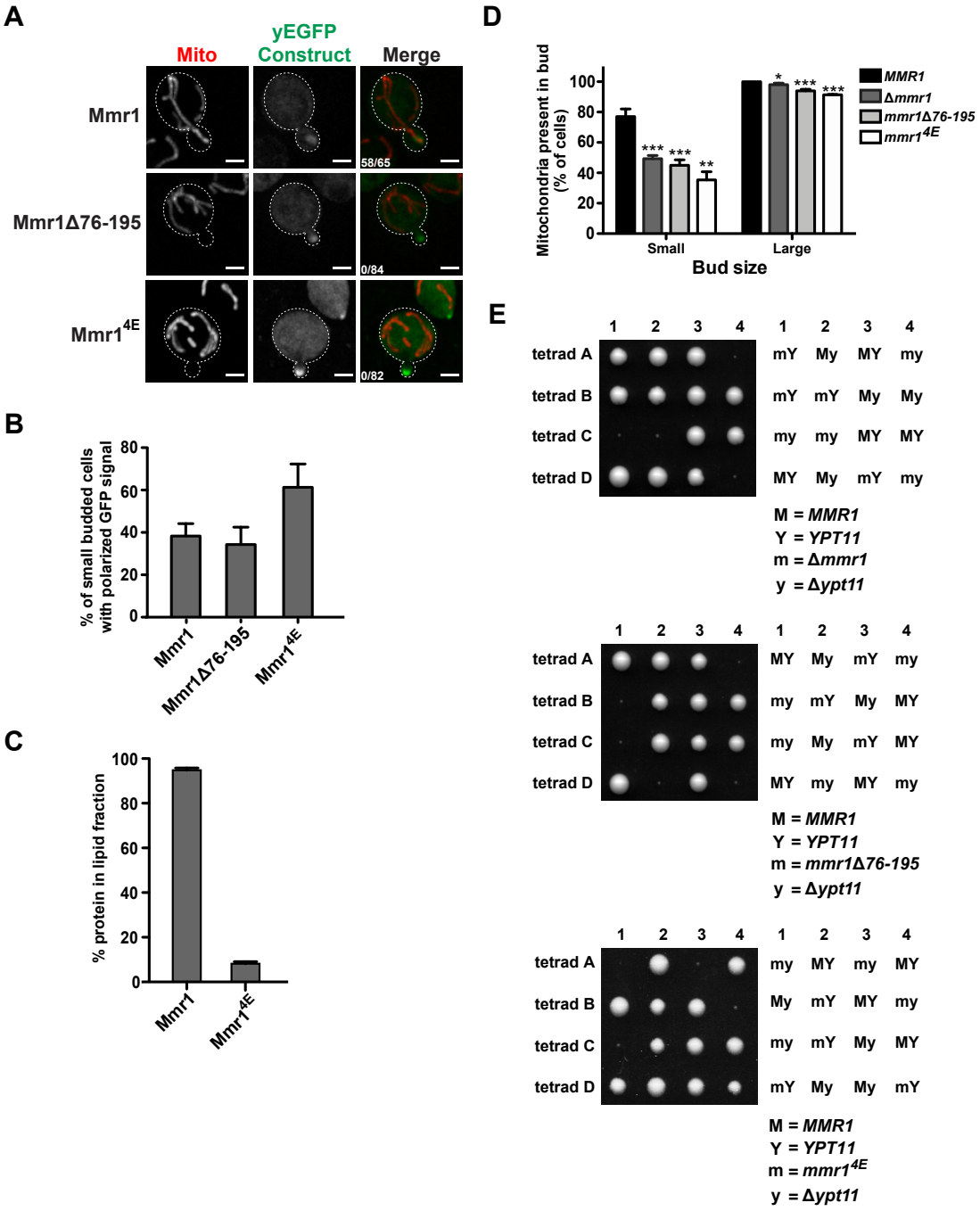
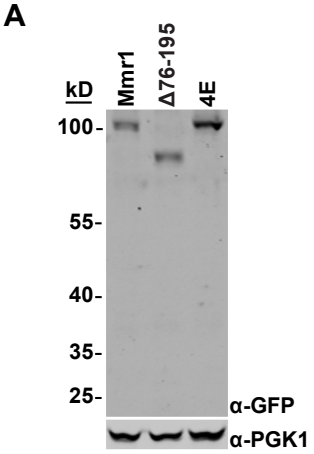


Figure 2.3: The membrane-binding domain of Mmr1 is required for Mmr1 function. (A, B) Cells expressing mitoRED and Mmr1-yEGFP, Mmr1Δ76–195-yEGFP, or Mmr1^{4E}-yEGFP were analyzed by fluorescence microscopy. Whole cell projections are shown in A. The cell cortex is outlined with a dashed white line. Scale bar, 2 μm. The number of cells in which the yEGFP fusion was observed to colocalize with mitochondria out of the total number of cells counted is shown in the bottom left corner of the merge image panel. Quantification of the polarized localization of the yEGFP fusion proteins in small-budded cells is shown as the mean ± SD in B; $n = 3$ independent experiments in which ≥78 small-budded cells were counted. (C) Purified Mmr1 and Mmr1^{4E} (5 μM) were incubated with OMC + 17% CL liposomes. The reactions were subjected to liposome flotation and analyzed as described in Figure 2.1A. Data are

Figure 2.3 (continue from previous page)
shown as the mean ± SEM; $n = 3$ independent experiments. (D) The presence of mitochondria in small and large buds was quantified in cells expressing wild-type Mmr1-yEGFP, Mmr1Δ76–195-yEGFP, and Mmr1^{4E}-yEGFP along with mitoRED. Buds were classified based on the bud-to-mother-diameter ratio: small buds have a bud/mother-diameter ratio of <1/3 and large buds have a bud/mother-diameter ratio of ≥1/3. Data are shown as the mean ± SD; $n = 3$ independent experiments in which ≥84 cells were counted for each bud size. p values are in comparison to *MMR1* cells of the comparable bud size. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. (E) Δ*ypt11*Δ*mmr1*, Δ*ypt11 mmr1*Δ*76–195-yEGFP*, and Δ*ypt11 mmr1*^{4E}-*yEGFP* diploid cells were sporulated, and spores from individual tetrads were arranged in a row on YPD medium. Growth on selective plates was used to score the markers for the deletions and yEGFP fusion and determine the genotypes of the haploid cells, which are indicated.



B

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SGD_Scer_MMR1/YLR190W       MNSPTMKSEQLTPKLSPMSFCLDDQRNAGSFQNLLNSPTKLKLDTGPIGNSLLYPTSLSK 60
MIT_Spar_c137_16122         MNSPTMKSEQLTPKLSPMSFCLDDQKNAGTFQNLLNSPTKLKLDTGSIGNSLLYPTSLSK 60
MIT_Suva_c324_17159         MNSPTMKSEQLTPKLSPMSFCLDDQRNANSFQNILNSPTKLKLDTGSIGNSLLYPTSLSK 60
WashU_Sbay_Contig504.6      MNSPTMKSEQLTPKLSPMSFCLDDQRNANSFQNILNSPTKLKLDTGSIGNSLLYPTSLSK 60
WashU_Skud_Contig2032.2     MNSPTMKSEQLTPKLSPMSFCLDDKKNAGSFQNLLNSPTKLKLETGSISNSLLYPTSLSK 60
WashU_Smik_Contig2325.2     MNSATMKSEQLTPKLSPMSFCLDDQKNAGTFQSILNSPTKLKLDTGPISNSLLYPTSLSK 60
                            *** ********************::**.:**.:*********:** *.***********

SGD_Scer_MMR1/YLR190W       LSELSRGGRSKQRRGSDTMRSVSPIRFQFLNNTPKMLKPEYLSQTTSNLPLLSALLKNSK 120
MIT_Spar_c137_16122         LSELSRNGRSKQRRGSDTMRSVSPIRFQFLNNTPKMLKPEYLSQTTNNLPLLSALLKNGK 120
MIT_Suva_c324_17159         LSELSRNGRSKQRRGSDTMRSVSPIRFQFFNNTPKMLKPEYLSQKSSGLPLLSALLKNSK 120
WashU_Sbay_Contig504.6      LSELSRNGRSKQRRGSDTMRSVSPIRFQFFNNTPKMLKPEYLSQKSSGLPLLSALLKNSK 120
WashU_Skud_Contig2032.2     LSELSRGGRSKQRRGSDTMRSVSPIRFQFLNNTPKMLKPEYLSQTTNNLPLLSALLKNSK 120
WashU_Smik_Contig2325.2     LSELSRSGRSKQRRGSDTMRSVSPIRFQFLNNTPKMLKPEYLSQTTNNLPLLSALLKNNK 120
                            ******.**********************:**************.:..**********.*

SGD_Scer_MMR1/YLR190W       KT----TSEGQNSNPDPLNIEKNIIKQSIKDKLEQLRS--SESVAQVQKKERNPPSFEAK 174
MIT_Spar_c137_16122         KT----TSEGQSPNPDPLNIEKNIIKQSIKDKLEQLRS--SESVTQVQKKDRKPASLEVK 174
MIT_Suva_c324_17159         QGSKSNSNEDQSVNPDPLSIEQNIVKQSIKDKLEQLRRQGSEQVAQIQKKEQQSTLPETK 180
WashU_Sbay_Contig504.6      QGSKSNSNEDQSVNPDPLSIEQNIVKQSIKDKLEQLRRQGSEQVAQIQKKEQQSTLPETK 180
WashU_Skud_Contig2032.2     KTPKANTGEDGSLNPDHLNIEKTIIKQTIKDKLEQLRN--AEHAVPVQNKEQKTMPLETI 178
WashU_Smik_Contig2325.2     KTTKSTTNEDQSLNPDPLSIEKNIIKQSIKDKLEQLRS--SEPISQVQKEERKPSPHDIK 178
                            :     :.*. . *** *.**:.*:**:*********   :*    :*:::::    :

SGD_Scer_MMR1/YLR190W       VCAEEPILRKNAEGLLPSYVPVPATPLEDPENHGVRKVEDKGLRVVSGGSTQCLSTEVNE 234
MIT_Spar_c137_16122         DCVEEPILRKNREGSVTSCVPASVIPLENTIGHGMQKVEDKGHRVISSGSTQCLSTEVSE 234
MIT_Suva_c324_17159         DYVEEPTAQKVSENSVCLY--TPEVPLEDTLDHVIPEVEDKENRFVSNGSTQCLNTEISD 238
WashU_Sbay_Contig504.6      DYVEEPTAQKVSENSVCLY--TPEVPLEDTLDHVIPEVEDKENRFVSNGSTQCLNTEISD 238
WashU_Skud_Contig2032.2     YYAEEPTPQEKPENSVSLYTTVPAPALE----------NHSMPSISNGSTHYSHTEVSE 227
WashU_Smik_Contig2325.2     DFTEETINQKNAESSGLLCTPVSVTPFEDTVSHEMPKAEDKGNRVISNGSTQCSQTEINE 238
                              .**   ::  *.       .    :*           ::    :*.***:   **:.:
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Figure 2.4: **Mmr1 mitoBD constructs.** (A) Whole cell extracts of the strains shown in Figure 2.3A were analyzed by SDS-PAGE and western blot using anti-GFP to detect the yEGFP fusion constructs and anti-PGK as a loading control. (B) An alignment of the first 234 amino acids of Mmr1 with homologs from other yeast species. The mitoBD of Mmr1 is shown in green, and the conserved residues that are mutated in Mmr1^{4E} are indicated with red triangles.

2.D Direct membrane binding contributes to Mmr1 function in mitochondrial inheritance

We next assessed mitochondrial inheritance in cells expressing Mmr1Δ76-195-yEGFP and Mmr1^{4E}-yEGFP. In the absence of Mmr1, a greater fraction of small-budded cells are devoid of mitochondria in comparison to wildtype cells, indicative of a delay in the inheritance of mitochondria (Itoh *et al*., 2004). We found that cells expressing Mmr1Δ76-195-yEGFP and Mmr1^{4E}-yEGFP exhibit a delay in mitochondrial inheritance similar to that observed for cells lacking Mmr1 (Figure 2.3D). In the absence of Mmr1, Myo2-driven mitochondrial inheritance is dependent on the Myo2 adaptor protein Ypt11, and in the absence of both Ypt11 and Mmr1, cells are inviable or severely impaired for growth (Itoh *et al*., 2004; Frederick *et al*., 2008; Chernyakov *et al*., 2013). Consistent with direct membrane binding of Mmr1 being critical for its function in mitochondrial inheritance, *mmr1Δ76-195* Δ*ypt11* and *mmr1^{4E}* Δ*ypt11* cells exhibited growth defects similar in severity to Δ*mmr1* Δ*ypt11* cells (Figure 2.3E). Together, these data indicate a direct interaction between Mmr1 and the mitochondrial membrane is critical for Mmr1 function in mitochondrial inheritance.

2.E The coiled-coil domain of Mmr1 is necessary and sufficient for Mmr1 self-interaction

Our structure-function analysis of Mmr1 identified an unpredicted membrane binding region within the protein, adding another functional domain to Mmr1 in addition to a well-characterized Myo2 binding domain and two putative PEST motifs (Figure 2.2D; Itoh *et al*., 2004; Eves *et al*., 2012). Mmr1 also contains a predicted coiled-coil (CC) domain (Itoh *et al*., 2004). While the CC domain of Mmr1 is suggested to be important for the function of the protein (Itoh *et al*., 2004), it is not clear how the CC domain contributes to Mmr1 function. Intermolecular self-interaction has been suggested to be necessary for the function of the mitochondrial tethering protein Num1 and

the Myo2 adaptor protein Smy1, and likely serves to increase the avidity of the proteins for their binding partners. For both Num1 and Smy1, the CC domains of the proteins mediate self-interaction (Tang *et al*., 2012; Lwin *et al*., 2016; Ping *et al*., 2016). To determine if the CC domain of Mmr1 mediates self-interaction, we examined the ability of Mmr1 to self-interact using a yeast two-hybrid assay conducted in Δ*mmr1* cells. Thus, the activation and binding domain fusions of Mmr1 were the only source of Mmr1 protein in the cells. We were able to detect an Mmr1-Mmr1 interaction in this assay (Figure 2.5A). Using a series of truncated Mmr1 constructs, we determined that the CC domain of Mmr1, amino acids 288-387, was sufficient for self-interaction (Figure 2.5A). Because the CC domain partially overlaps with the Myo2 binding domain, we wanted to create an Mmr1 construct that was able to interact with Myo2 but not with itself to test the functional significance of self-interaction. Therefore, we constructed Mmr1Δ288-377 (referred to as Mmr1ΔCC), in which the vast majority of the CC domain is deleted but the Myo2 binding domain is left intact (Eves *et al*., 2012). Indeed, this construct was able to interact with Myo2 but not itself, full length Mmr1, or Mmr1Δ76-195 (Figure 2.5B and C). These results suggest that the CC domain is necessary and sufficient for Mmr1 self-interaction but is not required for the interaction with Myo2.

To further test the idea that the CC domain mediates Mmr1 self-interaction, we examined the ability of Mmr1ΔCC to self-interact in cells using co-immunoprecipitation assays. We coexpressed differentially tagged versions of Mmr1ΔCC in diploid cells and examined the ability of Mmr1ΔCC-FLAG to co-immunoprecipitate Mmr1ΔCC-yEGFP. In comparison to the steady-state levels of wildtype Mmr1-FLAG and Mmr1-yEGFP, the steady-state levels of Mmr1ΔCC-

FLAG and Mmr1ΔCC-yEGFP expressed from the endogenous *MMR1* promoter were increased (Figure 2.5D, lysate).

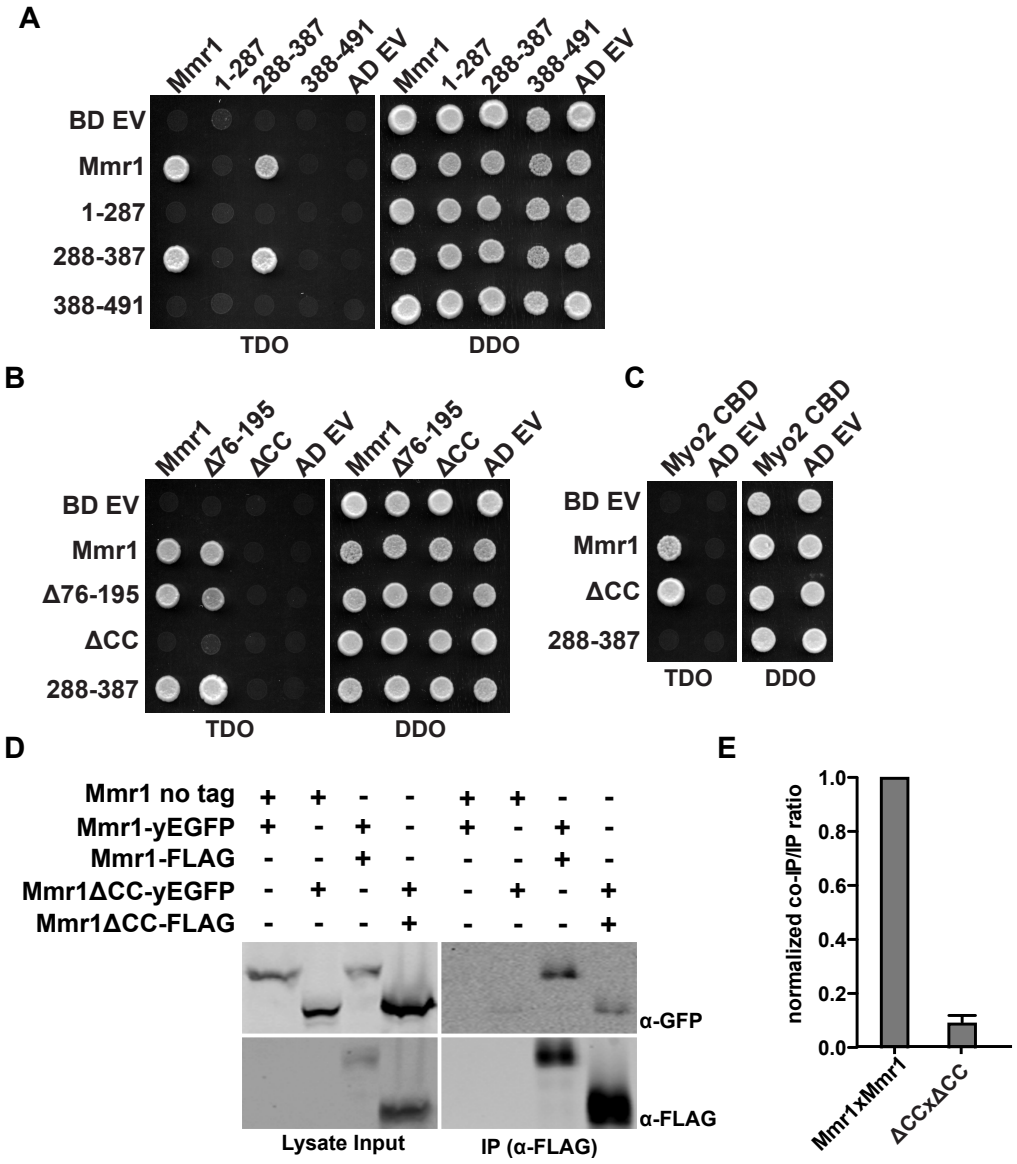

Figure 2.5: Mmr1 self-interaction is mediated by the CC domain. (A–C) Yeast two-hybrid assays to assess interactions between the indicated regions of Mmr1 (A), the ability of Mmr1Δ76–195 and Mmr1ΔCC to self-interact (B), and interactions between Mmr1ΔCC and the Myo2 cargo-binding domain (CBD) (C). For all yeast two-hybrid assays, protein–protein interactions were assessed by growth on triple-dropout (TDO) medium. AD EV, activation domain empty vector; BD EV, binding domain empty vector; TDO, SC–Leu–Trp–Ade; double-dropout medium (DDO), SC–Leu–Trp. (D, E) Cell lysates from diploid cells expressing Mmr1-FLAG and Mmr1-yEGFP or Mmr1ΔCC-FLAG and Mmr1ΔCC-yEGFP were subjected to anti-FLAG immunoprecipitation (IP). Cell lysates and IP elutions were analyzed by SDS–PAGE and Western blot using anti-FLAG and anti-GFP antibodies (D). Quantification of the normalized co-IP/IP ratio is shown in E as the mean ± SD, $n = 3$ independent experiments.

Despite the increased levels of the Mmr1ΔCC proteins, the ability of Mmr1ΔCC-FLAG to co-immunoprecipitate Mmr1ΔCC-yEGFP was dramatically reduced compared to the ability of Mmr1-FLAG to co-immunoprecipitate Mmr1-yEGFP (Figure 2.5D and E). These results further support the idea that the CC domain of Mmr1 mediates Mmr1 self-interaction.

2.F Self-interaction contributes to Mmr1 function in mitochondrial inheritance

To test the functional significance of Mmr1 self-interaction, we examined the localization of Mmr1ΔCC-yEGFP and mitochondrial inheritance in these cells. In comparison to the punctate, bud-enriched localization of Mmr1-yEGFP, Mmr1ΔCC-yEGFP localized more evenly along mitochondria in both the mother and bud with less bud enrichment (Figure 2.6A). Thus, the CC domain is required for the proper distribution of Mmr1 within cells but is not required for the association with mitochondria. Cells expressing Mmr1ΔCC also exhibited subtle, non-significant defects in mitochondrial inheritance in otherwise wildtype and Δ*ypt11* backgrounds (Figure 2.6B), suggesting the function of Mmr1ΔCC in mitochondrial inheritance may be attenuated.

Based on our co-immunoprecipitation experiments, we noted that the levels of Mmr1ΔCC-yEGFP were higher than wildtype Mmr1-yEGFP. Indeed, the steady-state protein levels of Mmr1ΔCC-yEGFP were found to be ~16x that of Mmr1-yEGFP (Figure 2.7A and C).

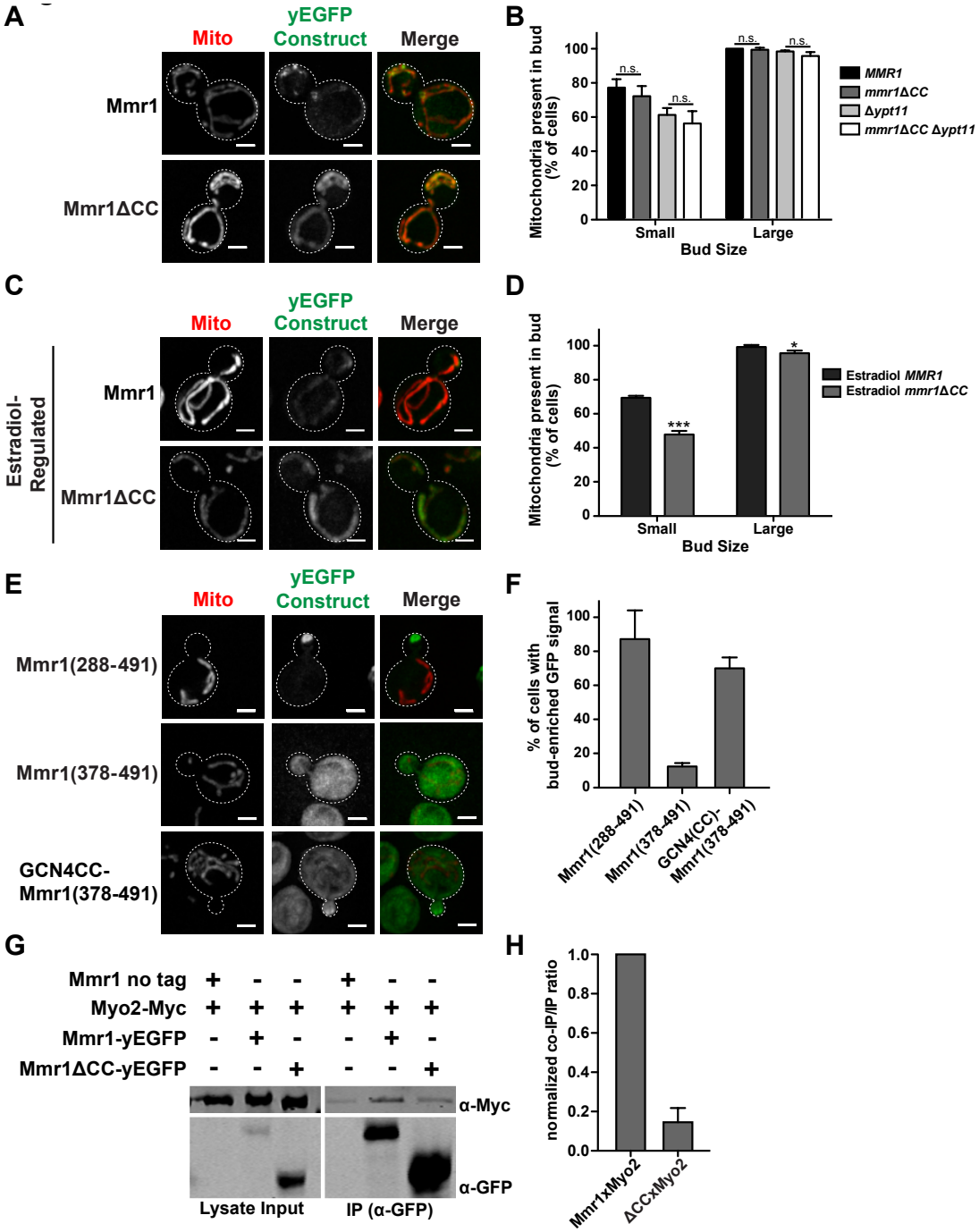

Figure 2.6: The CC domain of Mmr1 is required for Mmr1 function. (A, B) Cells expressing mitoRED and Mmr1-yEGFP and Mmr1ΔCC-yEGFP, as indicated, were analyzed by fluorescence microscopy (A), and the presence of mitochondria in small and large buds was quantified as described in Figure 2.3D (B). Whole cell projections are shown. The cell cortex is outlined with a dashed white line. Scale bar, 2 μm. Data are shown as the mean ± SD; n = 3 independent experiments in which ≥86 cells were counted for each bud size. n.s., not significant. (C, D) Cells expressing mitoRED and Mmr1-yEGFP or Mmr1ΔCC-yEGFP from an estradiol-regulated GalS promoter were grown in the presence of 0.5 and 0.05 nM estradiol, respectively. The cells were analyzed by fluorescence microscopy (C), and the presence of mitochondria in small and large buds was quantified as described in Figure 2.3D (D). Whole cell projections are shown. The cell cortex is outlined with a dashed white line. Scale bar, 2 μm. Data are

Figure 2.6 (continue from previous page)
shown as the mean ± SD; $n = 3$ independent experiments in which ≥86 cells were counted for each bud size. p values are in comparison to estradiol *MMR1* cells of the comparable bud size. ***, $p < 0.001$; *, $p < 0.05$. (E, F) Cells expressing mitoRED and Mmr1(288–491)-yEGFP, Mmr1(378–491)-yEGFP, or GCN4CC-Mmr1(378–491), as indicated, were analyzed by fluorescence microscopy. Whole cell projections are shown in E. The cell cortex is outlined with a dashed white line. Scale bar, 2 μm. Quantification of small-budded cells with a bud-enriched localization of the yEGFP fusion protein is shown as the mean ± SD in F; $n = 3$ independent experiments in which ≥79 small-budded cells were counted. Any cell with an enrichment of the protein at the bud tip above the cytosolic signal was counted as bud-enriched. (G, H) Cell lysates from cells expressing Myo2-Myc and either Mmr1-yEGFP or Mmr1ΔCC-yEGFP were subjected to anti-GFP immunoprecipitation (IP). Cell lysates and IP elutions were analyzed by SDS–PAGE and Western blot using anti-GFP and anti-Myc antibodies. Quantification of the normalized co-IP/IP ratio is shown in H as the mean ± SD, $n = 3$ independent experiments.

These results are consistent with the previous finding that disrupting the polarized localization of Mmr1 results in higher steady-state levels of the protein (Eves *et al*., 2012). In addition, these results raise the possibility that overexpression of the protein may be compensating for its attenuated function, decreasing the severity of the phenotypes observed. Therefore, we sought to examine the function of Mmr1ΔCC-yEGFP when expressed at levels more similar to wildtype Mmr1. To this end, we placed a GalS promoter upstream of *MMR1-yEGFP* and *MMR1ΔCC-yEGFP* and engineered the strains to express a transcription factor that drives expression from the Gal promoter only in the presence of estradiol. The concentrations of estradiol were optimized so that the steady-state levels of Mmr1-yEGFP and Mmr1ΔCC-yEGFP were comparable to Mmr1-yEGFP expressed from the endogenous *MMR1* promoter (Figure 2.6C and 2.7B and C). Cells expressing Mmr1-yEGFP from the estradiol-regulated GalS promoter inherited mitochondria similarly to cells expressing Mmr1-yEGFP from the endogenous Mmr1 promoter (Figure 2.6B and D). In contrast, cells expressing Mmr1ΔCC-yEGFP from the estradiol-regulated GalS promoter exhibited a defect in mitochondrial inheritance similar in severity to that observed in Δ*mmr1* cells (Figure 2.3D and 2.6D). These results indicate that, at wildtype levels of Mmr1, the CC domain is critical for Mmr1-mediated mitochondrial

inheritance, and the function of the CC domain can be bypassed by overexpression of the protein.

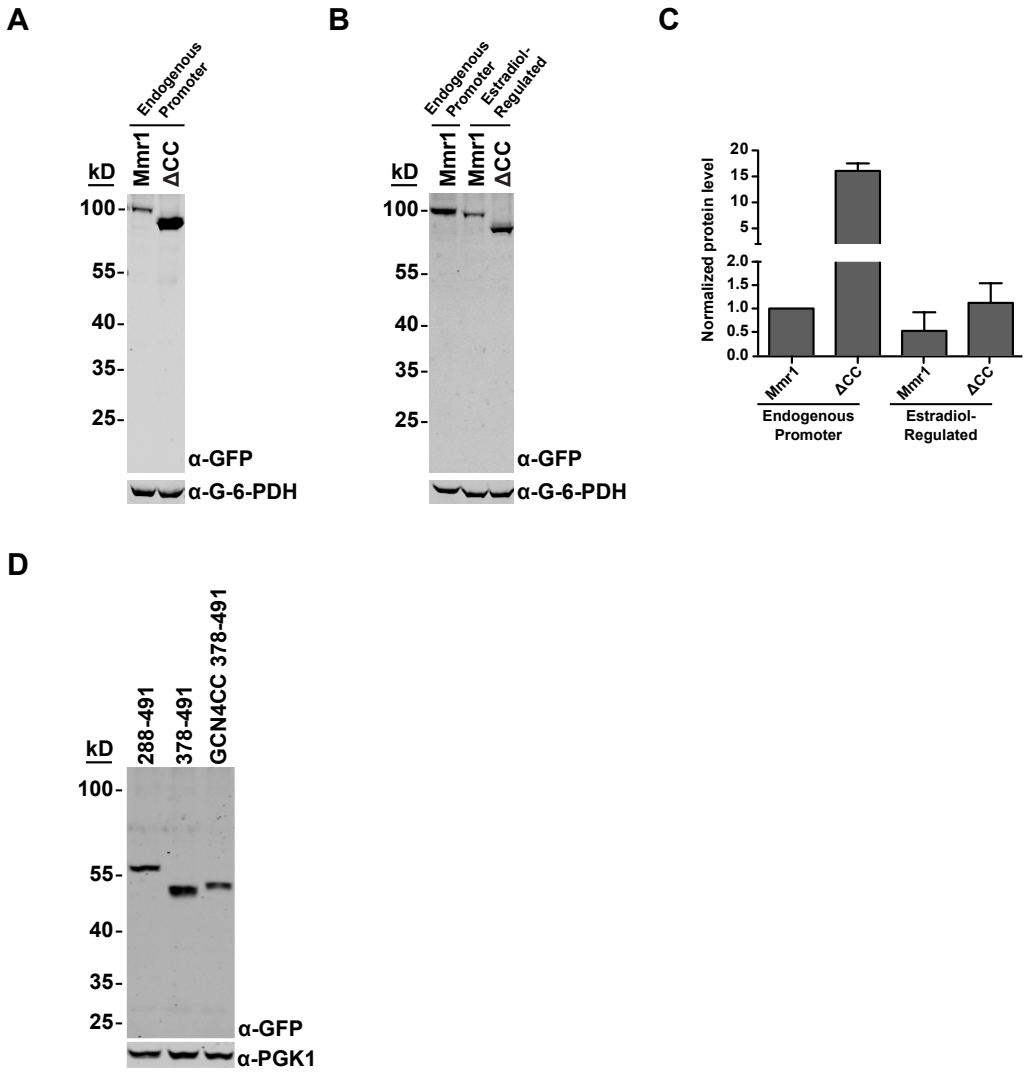

Figure 2.7: Protein levels of Mmr1 constructs. (A) Whole cell extracts of strains expressing Mmr1-yEGFP and Mmr1ΔCC-yEGFP from the endogenous MMR1 promoter, as indicated, were analyzed by SDS-PAGE and western blot using anti-GFP to detect the Mmr1-yEGFP truncations and anti-G-6-PDH as a loading control. (B and C) Whole cell extracts of strains expressing wildtype Mmr1-yEGFP from the endogenous promoter as well as Mmr1-yEGFP and Mmr1ΔCC-yEGFP from the estradiol-regulated GalS promoter, as indicated, were analyzed as in Fig. 2.7A. Quantification of the western blots is shown in C as the mean ± SD, n = 3 independent experiments. (D) Whole cell extracts of strains expressing Mmr1(288-491)-yEGFP, Mmr1(378-491)-yEGFP, GCN4CC-Mmr1(378-491)-yEGFP from the endogenous MMR1 promoter, as indicated, were analyzed as in Fig. 2.7A with exception to using anti-PGK as the loading control.

Interestingly, we found that the CC domain was also necessary for the polarized localization of the Mmr1 Myo2 BD. Specifically, we expressed Mmr1(288-491), which contains both the CC domain and Myo2 BD, and Mmr1(378-491), which contains only the Myo2 BD, as yEGFP fusions (Figure 2.7D). In contrast to the striking bud-polarized localization of Mmr1(288-491)-yEGFP, Mmr1(378-491)-yEGFP was evenly distributed in the cytosol of the mother and bud (Fig. 2.6E and F), consistent with previous findings (Itoh *et al*., 2004). To test if CC-mediated dimerization specifically was necessary for the polarized localization of the Mmr1 Myo2 BD, we replaced amino acids 288-377 of Mmr1 with the CC domain of GCN4, which forms a well characterized homodimer (O'Shea *et al*., 1991). Notably, the addition of the GCN4CC to Mmr1(378-491)-yEGFP partially restored the bud-polarized localization of the protein (Figure 2.6E and F). In contrast to the localization of Mmr1(288-491)-yEGFP, which robustly accumulated at the bud tip of small budded cells, GCN4CC-Mmr1(378-491)-yEGFP localized diffusely in the cytosol as well as in accumulations at the bud tip. The percentage cells with an enrichment of the protein at the bud tip was similar for both Mmr1(288-491)-yEGFP and GCN4CC-Mmr1(378-491)-yEGFP (Figure 2.6F). These results suggest that, even though constructs lacking the CC domain can interact with Myo2 (Figure 2.5C; Eves *et al*., 2012), dimerization driven by the CC domain plays a role in the Myo2-dependent polarization of the protein. To further test the idea that CC-mediated dimerization of Mmr1 enhances the interaction with Myo2, we examined the interaction between Mmr1ΔCC and Myo2 in cells using co-immunoprecipitation assays. We found that despite the increased levels of Mmr1ΔCC in cells, the ability of Mmr1ΔCC to co-immunoprecipitate Myo2 was reduced compared to the ability of Mmr1 to co-immunoprecipitate Myo2 (Figure 2.6G and H). Together, our results suggest that

self-interaction mediated by the CC domain is necessary for Mmr1 function in mitochondrial inheritance and likely functions to enhance the interaction between Mmr1 and Myo2.

2.G Conclusions and Discussion

Here we provide evidence demonstrating that direct membrane binding and self-interaction are critical for Mmr1 function in mitochondrial inheritance. Interestingly, these functional features are shared between Mmr1 and Num1, the core protein component of MECA. Both proteins exhibit the ability to directly interact with phospholipid membranes via unpredicted lipid binding domains and the ability to self-assemble (Tang *et al*., 2012; Ping *et al*., 2016). As proposed for Num1 (Kraft and Lackner, 2017), self-assembly of Mmr1 likely increases the avidity between Mmr1 and its binding partners. Indeed, our data suggest that Mmr1 self-interaction facilitates a robust interaction between Mmr1 and Myo2. The finding that overexpression of Mmr1ΔCC bypasses the function of the CC domain is consistent with the idea that the CC domain and Mmr1 self-interaction are not required for the interaction with Myo2 but enhance the interaction. Self-interaction likely also enhances the interaction between Mmr1 and mitochondria by increasing the number of membrane binding sites per functional unit and, therefore, the avidity of Mmr1 for the membrane. In contrast to a previous study that includes the CC domain as part of the mitochondrial and Myo2 binding domains (Itoh *et al*., 2004), our data indicate that the CC domain is not required for Mmr1-mitochondria and Mmr1-Myo2 interactions but instead likely impacts the robustness of these interactions. Two conserved residues in the mitochondrial binding domain and one conserved residue in the CC domain of Mmr1 have been identified as sites of phosphorylation (Swaney *et al*., 2013). We predict the spatial and temporal regulation of

phosphorylation at these sites will serve as a mechanism to regulate Mmr1-binding partner interactions and, consequently, Mmr1 function in space and time.

Similar to Num1 (Ping *et al*., 2016), in vitro Mmr1 and the Mmr1 mitoBD preferentially bind phospholipid membranes enriched in CL and also show preferential binding to PA. Like CL, PA is a negatively charged, cone-shaped lipid. Cone-shaped, or non-bilayer lipids, induce membrane curvature or create distinct microenvironments in a planar bilayer (van den Brink-van der Laan *et al*., 2004; Osman *et al*., 2011). Therefore, we speculate that Mmr1, rather than recognizing a specific phospholipid, recognizes a membrane structure that is formed or reinforced by CL, PA, and likely other lipid and protein factors. Consistent with this idea, noticeable defects in the association of Mmr1 or the Mmr1 mitoBD with mitochondria are not observed in cells that lack CL (Figure 2.8A and B). In addition, genetic interactions between *ypt11* and CL synthesis mutants are not observed (Figure 2.8C). Additional factors have been proposed to compensate for the lack of CL when CL synthesis is disrupted. For example, PE, a cone-shaped but neutral lipid, as well as PG, a CL precursor, have overlapping functions with and can substitute for CL (Chang *et al*., 1998; Gohil *et al*., 2005; Joshi *et al*., 2012). In cells lacking Ups1, a protein that functions early in the CL synthesis pathway, CL levels decrease and PA levels increase, most notably at contact sites between the outer and inner membranes (Connerth *et al*., 2012). The idea that other factors can compensate for CL is further supported by the finding that Mgm1, a protein that drives the fusion of mitochondria, preferentially bind to CL in vitro and its activity is stimulated by CL in vitro, but CL is not essential for mitochondrial fusion in cells (DeVay *et al*., 2009; Chen *et al*., 2010; Joshi *et al*., 2012). Thus, multiple lines of evidence suggest additional factors can compensate for the lack of CL in cells. While our data indicate that Mmr1 can

directly bind phospholipid membranes and that direct membrane binding contributes to Mmr1 function, we cannot exclude the possibility that mitochondrial proteins may contribute to the Mmr1-mitochondria interaction.

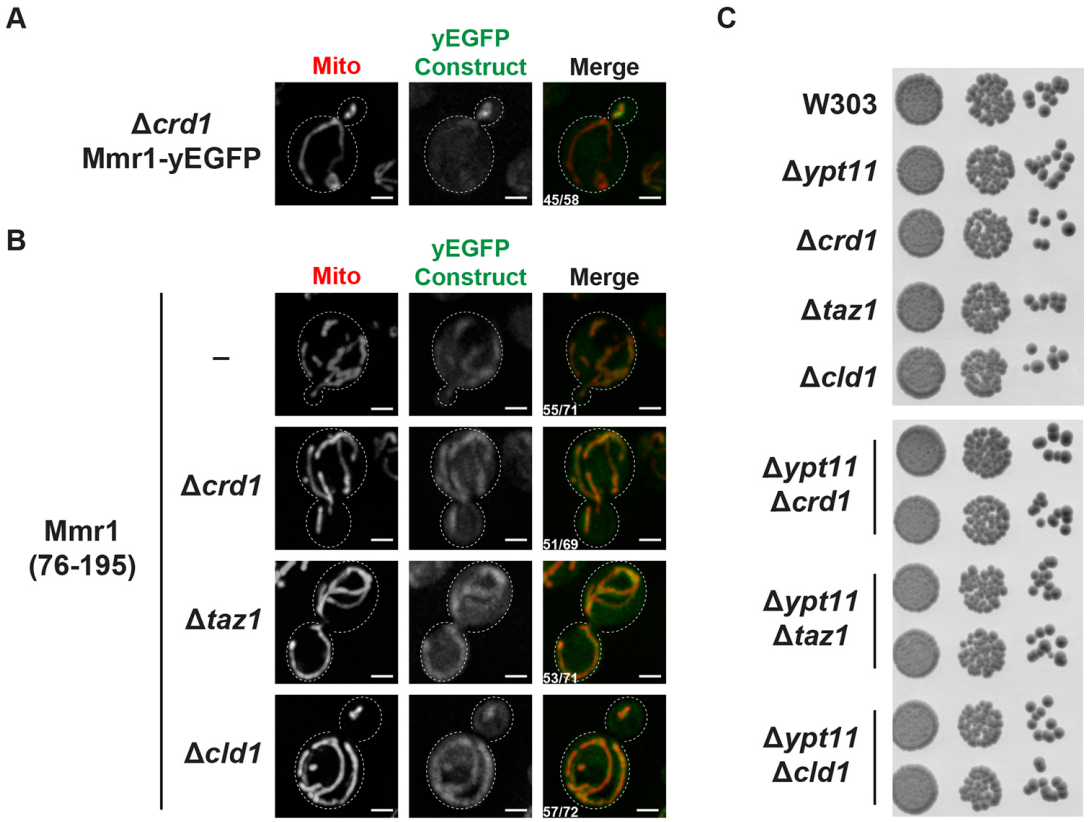

Figure 2.8: The effects of cardiolipin biosynthesis mutants on Mmr1 localization and function. (A and B) Δ*crd1* cells expressing Mmr1-yEGFP (A) and otherwise wildtype, Δ*crd1*, Δ*cld1*, or Δ*taz1* cells expressing Mmr1(76-195)-yEGFP (B) along with mitoRED were analyzed by fluorescence microscopy. Whole cell projections are shown. The cell cortex is outlined with a dashed white line. Bar, 2 μm. The number of cells in which the yEGFP fusion was observed to co-localize with mitochondria out of the total number of cells counted is shown in the lower left corner of the merge image panel. For Mmr1(76-195)-yEGFP in an otherwise wildtype background, this number is reproduced from Fig. 2.2A for ease of comparison. (C) Serial dilutions of the indicated strains were grown at 24°C on YPD.

Interestingly, the relationship between Mmr1 and Num1 function in mitochondrial positioning changes over the course of the cell cycle. Our previous work demonstrates that mitochondria drive the assembly of Num1 clusters. In the absence of mitochondrial inheritance, Num1 clusters do not form in buds. The lack of Num1 clusters not only disrupts mitochondria-plasma

membrane anchoring in large buds but also Num1-mediated dynein anchoring and, consequently, dynein-mediated spindle positioning (Kraft and Lackner, 2017). Therefore, what starts out as an antagonistic relationship between Mmr1 function in the bud and Num1 function in the mother early in the cell cycle turns into a facilitatory relationship in which the anchoring functions of Num1 in large buds are positively impacted by Mmr1-mediated mitochondrial inheritance later in the cell cycle. Therefore, the spatial and temporal regulation of Mmr1 function not only impacts mitochondrial positioning but also the formation of a mitochondria-plasma membrane anchor that functions in dynein-mediated nuclear positioning. In this context, we speculate that the functional connections and dependencies between two mitochondrial positioning pathways and a nuclear positioning pathway provide a means to order and integrate major spatial organization pathways within the cell.

Chapter 3

The function of Mmr1 in maintaining mtDNA integrity over generations

3.A Introduction

Commonly known as the powerhouse of the cell, mitochondria produce energy for the cell via oxidative phosphorylation. Oxidative phosphorylation utilizes the electron transport chain (ETC), which is a collection of protein complexes (Complex I-IV) that reside in the inner mitochondrial membrane. The ETC utilizes the energy released by transferring electrons through the complexes to build up an electrochemical gradient across the inner mitochondrial membrane. This electrochemical gradient is harnessed by the ATP synthase complex to produce ATP, a form of chemical energy used by the cell. Most of the proteins in the electron transport chain are encoded by the nuclear genome and get imported into the mitochondria; however, some essential subunits of the ETC complexes are encoded by the mitochondrial genome (mtDNA). Therefore, mtDNA is critical for respiratory growth, which requires oxidative phosphorylation, in all eukaryotic cells. Mutations within mtDNA cause cellular growth defects in yeast and result in many human diseases including neurodegenerative diseases, metabolic diseases and various types of cancer (Taylor *et al*., 2005).

In the budding yeast *Saccharomyces cerevisiae*, mtDNA is 85.8 kb long and is packaged by mtDNA-binding proteins into nucleoprotein complexes, termed nucleoids. Each nucleoid contains 1-10 mtDNA copies (Chen *et al*., 2005 Lipinski *et al*., 2010) with each DNA copy encoding seven essential subunits of the ETC complexes. In most eukaryotic cells, nucleoids exist in multiple copies and are distributed throughout the mitochondrial network. In yeast for example, nucleoids are maintained at ~50-200 copies and are evenly distributed with a consistently observed distance of ~800 nm (Osman *et al*., 2015).

During cell division, mitochondria, including nucleoids, need to be faithfully segregated into both resulting cells. Specifically, during asymmetric cell division in budding yeast and stem

cells, mitochondria are thought to be selectively partitioned into the two resulting cells where one cell obtains higher functioning mitochondria than the other. This asymmetric partitioning of mitochondria has been shown to play an important role in giving rise to the asymmetric characteristics of the dividing cells (Katajisto *et al*., 2015, Pernice *et al*., 2016). Studies across these cell types have focused on understanding the process by which the mitochondrial compartment is inherited. Studies in budding yeast have shown two mitochondrial adaptors, Mmr1 and Ypt11, are important for mitochondrial inheritance. These mitochondrial adaptors function by interacting with the motor protein, Myo2, to traffic mitochondria via actin cables. Work from our lab and others has provided mechanistic insight into how these adaptors contribute to the inheritance of mitochondria. However, the question of how nucleoids containing functional mtDNA are inherited is still unknown. Research has demonstrated that mtDNA is often associated with the tips of mitochondrial tubules that are invading the bud (Osman *et al*., 2015). However, the mechanisms that ensure nucleoids are faithfully localized to the mitochondrial tip are unknown. In addition, it is not known if, and how, functional mtDNA is selected for inheritance.

Mmr1 has been suggested to be essential for the inheritance of higher functioning mitochondria by the bud as measured by higher membrane and redox potential and lower levels of reactive oxygen species（Higuchi-Sanabria *et al*., 2014). Each of these readouts of mitochondrial function assesses the function of the electron transport chain. Key components of the ETC complexes are encoded by mtDNA, suggesting that higher functioning mitochondria are associated with functional mtDNA. Therefore, we hypothesize Mmr1 plays a role in selecting mitochondria that contain functional mtDNA for transport to daughter cells. Here we assess the effects of deleting *MMR1* on mtDNA inheritance and the effect mtDNA has on mitochondrial

inheritance. We find Δ*mmr1* cells lose mtDNA integrity; we observe an increase in the number of cells either lacking mtDNA or containing non-functional mtDNA. In addition, cells lacking mtDNA have a subtle delay in mitochondrial inheritance, suggesting a connection between mtDNA and mechanisms of mitochondrial inheritance. Interestingly we find that the contribution of Mmr1 to mitochondrial inheritance is reduced in respiratory conditions. Together our results suggest that in addition to its role in inheritance of the mitochondrial compartment, Mmr1 plays a role in maintaining mtDNA integrity over generations.

3.B Mmr1, not Ypt11, is important for maintaining functional mtDNA

Mmr1 functions in the Myo2-dependent transport of mitochondria into the growing bud and also anchors mitochondria at the bud tip. Ypt11, a Rab GTPase protein, serves as a partially redundant mitochondrial adaptor for Myo2. Although both adaptors function in Myo2-dependent mitochondrial inheritance, it is not known whether either adaptor plays a role in the inheritance of mtDNA. To test whether Mmr1 or Ypt11 function in mtDNA inheritance, we examined the ability of cells to produce daughters that contain functional mtDNA in the absence of Mmr1 or Ypt11 using the petite frequency assay. The petite frequency assay is carried out as follows: 1) We initially select for cells with functional mtDNA by growing them in media containing a carbon source that forces cells to respire, ethanol glycerol (EG). 2) We remove the selection for functional mtDNA by growing cells in media containing a fermentable carbon source, dextrose (Dex), for a defined period of time. 3) Cells are then plated on rich media Dex plates that contain low amounts of adenine. The cells that lose functional mtDNA will appear as white, petite colonies on the low adenine plates. These white, petite colonies are attributed to defects in mitochondrial function because the W303 strain background used has a defect in the adenine

synthesis pathway, *ade2-1*. On low adenine plates, cells with functional mitochondria will appear red due to the accumulation of a red adenine intermediate in the vacuole. However, cells that lack functional mitochondria will appear white due to the lack of intermediate accumulating in the vacuole (Bharathi *et al*., 2016).

The petite frequency assay was performed on wildtype, Δ*mmr1*, and Δ*ypt11* cells grown in Dex conditions for 48 hours. Wildtype and Δ*ypt11* cells exhibit only ~15% petite colonies, while Δ*mmr1* cells exhibit ~60% petite colonies (Figure 3.1A). These data suggest Mmr1 either plays a role in the maintenance or inheritance of mtDNA given that the deletion of *MMR1* leads to a four-fold increase in petite colonies. Interestingly, this increase in petite colonies is not observed in Δ*ypt11* cells, suggesting that Ypt11 is dispensable for maintaining mtDNA over multiple generations.

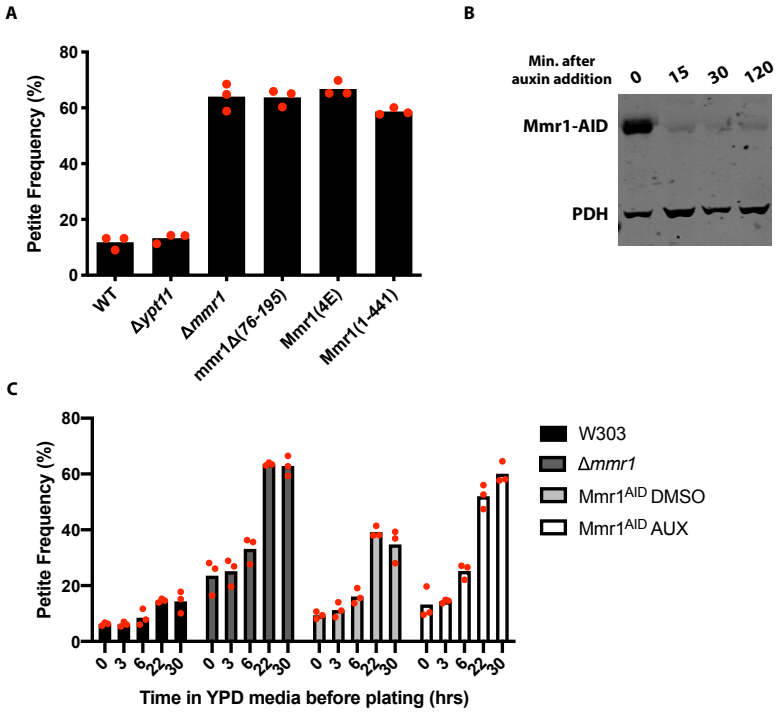

Figure 3.1 Mmr1, not Ypt11, is important for maintaining functional mtDNA.
(A) The presence of petite colonies in wildtype, Δ*ypt11,* Δ*mmr1*, Mmr1Δ(*76–195*), Mmr1(4E) and Mmr1(1-441) cultures after growing in dextrose for 48 hours was quantified. (B) Whole cell extracts of a strain expressing Mmr1-FLAG from the endogenous *MMR1* promoter were grown in ethanol glycerol conditions and 0, 15, 30, 120 mins after auxin addition, as indicated, were analyzed by SDS-PAGE and

Figure 3.1 (continue from previous page)
western blot using anti-FLAG to detect the Mmr1-FLAG truncations and anti-G-6-PDH as a loading control. (C) The presence of petite colonies for wildtype, Δ*mmr1*, and *mitoAID* grown with or without auxin after growing cells in dextrose for 0, 6, 22, 30 hours.

We and others have previously shown that the interactions of Mmr1 with Myo2 and mitochondria are required for Mmr1-dependent mitochondrial inheritance. To elucidate whether the role of Mmr1 in mitochondrial transport is required for its function in the maintenance of mtDNA, we examined the importance of the Mmr1-mitochondria and Mmr1-Myo2 interactions in maintaining functional mtDNA over generations. We examined the petite frequency of cells expressing Mmr1 mutants that are unable to interact with mitochondria or Myo2. We find that mutants that are unable to interact with mitochondria, Mmr1Δ76-195 and Mmr1(4E), and Myo2, Mmr1(1-441), exhibit a petite frequency similar to Δ*mmr1* cells after growing in fermentation conditions for 48 hours, suggesting Mmr1-dependent transport is essential for the maintenance of mtDNA (Figure 3.1A).

To examine the rate at which Δ*mmr1* cells go petite, we quantified the petite frequency of wildtype and Δ*mmr1* cells over time. The basal petite frequency (t=0) of Δ*mmr1* cells is ~22% compared to 7% for wildtype cells, and we see wildtype cells accumulate petite colonies at a slower rate compared to Δ*mmr1* cells (Figure 3.1C). To watch the petite phenotype of *mmr1* cells evolve, we used the auxin-inducible degradation system to rapidly induce the degradation of Mmr1. In order to construct Mmr1^{AID}, we tagged *MMR1* with the AID-FLAG tag at its endogenous locus in wildtype cells expressing Tir1, a plant-specific F-box protein. This strain is referred to as Mmr1^{AID}. Tir1 binds the yeast SCF (Skp1, Cullen, F-box) complex and, in the presence of auxin, recruits the SCF complex to AID-tagged proteins, which are subsequently ubiquitinated and targeted for proteasomal degradation (Nishimura *et al*., 2009). Upon the addition of auxin, Mmr1^{AID} can be conditionally depleted in 15 mins (Figure 3.1B). At t=0,

Mmr1^{AID} cells exhibit a petite frequency similar to wildtype cells (Figure 3.1C). Over time, Mmr1^{AID} under auxin condition show accumulation of petite colonies similar to Δ*mmr1* (Figure 3.1C). However, in the absence of auxin, Mmr1^{AID} cells also have a higher petite frequency than wildtype but lower than Mmr1^{AID} cells in the presence of auxin and Δ*mmr1* cells. These results suggest the addition of AID-FLAG tag may compromise Mmr1's function or the AID system is leaky resulting in some Mmr1 degradation in the absence of auxin. Overall, these results suggest that depletion of Mmr1 affects either the maintenance or the inheritance of mtDNA.

3.C Δ*mmr1* cells exhibit an increased frequency of mtDNA mutation and loss

Next, we asked whether the respiration deficient colonies produced by Δ*mmr1* cells lack mtDNA or contain non-functional mtDNA. Using DAPI stain, we were able to visualize mtDNA under the fluorescence microscope and found that out of 13 petite colonies examined, 23% were devoid of mtDNA and 77% contained mtDNA (Figure 3.2A). These data suggest that Δ*mmr1* cells contain a mixed population of petite colonies, those with non-functional mtDNA (rho$^-$ cells) and those completely devoid of mtDNA (rho^0 cells). We used PCR analysis to confirm these results. We PCR amplified *COX3*, *COB* and *ATP9* as a readout of the presence of mtDNA using the same cells that were used for the visual analysis by DAPI (Figure 3.2B). The three genes are located in different regions of the mitochondrial genome and are essential for cellular respiration. For wildtype cells, we were able to amplify all three genes. For rho$^-$ cells, we are able to amplify a subset of the three genes, and for rho^0 cells, we are unable to amplify any of the genes (Figure 3.2 C). These results further support the finding that Δ*mmr1* cells exhibit an increased frequency of mtDNA mutation and loss.

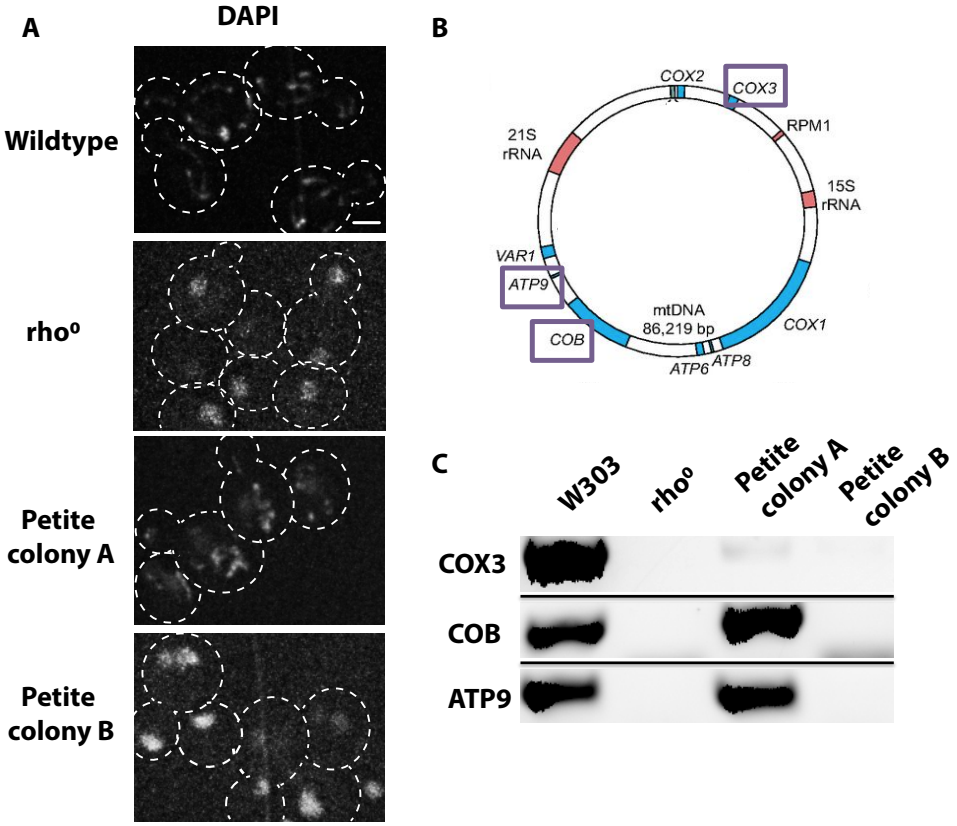

Figure 3.2: Δ*mmr1* cells contain a mixed population of rho$^-$ and rho^0 cells. (A) Wildtype cells, rho^0 cells, petite colony A cells or petite colony B cells, as indicated, from the 48 hour petite frequency assay were DAPI stained and analyzed by fluorescence microscopy. (B) Schematic of mtDNA with purple boxes around those genes that are amplified in C. (C) *ATP9*, *COB*, *COX3* genes encoded by the mitochondrial genome are PCR amplified in wildtype cells, rho^0 cells, and cell from petite colony A and B.

3.D The lack of mtDNA affects proper mitochondrial inheritance

Based on our findings thus far, Mmr1 plays a critical role in the maintenance of functional mtDNA over generations. It is possible that Mmr1 preferentially interacts with mitochondria that contain mtDNA to ensure mtDNA is inherited by daughters. If so, then eliminating mtDNA should disrupt Mmr1-dependent mitochondria inheritance. To test the impact of mtDNA on Mmr1-dependent mitochondrial inheritance, we examined the function and localization of Mmr1 in cells depleted of mtDNA. Ethidium bromide (EtBr) was used to eliminate mtDNA and, therefore, create rho^0 cells. Mmr1 and mitochondria were simultaneously

imaged in rho^0 cells, and Mmr1 localization did not appear to be altered in rho^0 cells (Figure 3.3A). However, rho^0 cells did have a slight mitochondrial inheritance delay compared to wildtype cells, but the defect was not as severe as Δ*mmr1* cells. This slight inheritance delay is likely not due to morphological defects, as mitochondrial morphology in rho^0 cells remains tubular. This suggests that mtDNA does impact mitochondrial transport. However, if the effect of mtDNA on mitochondrial inheritance is direct or indirect still remains unknown.

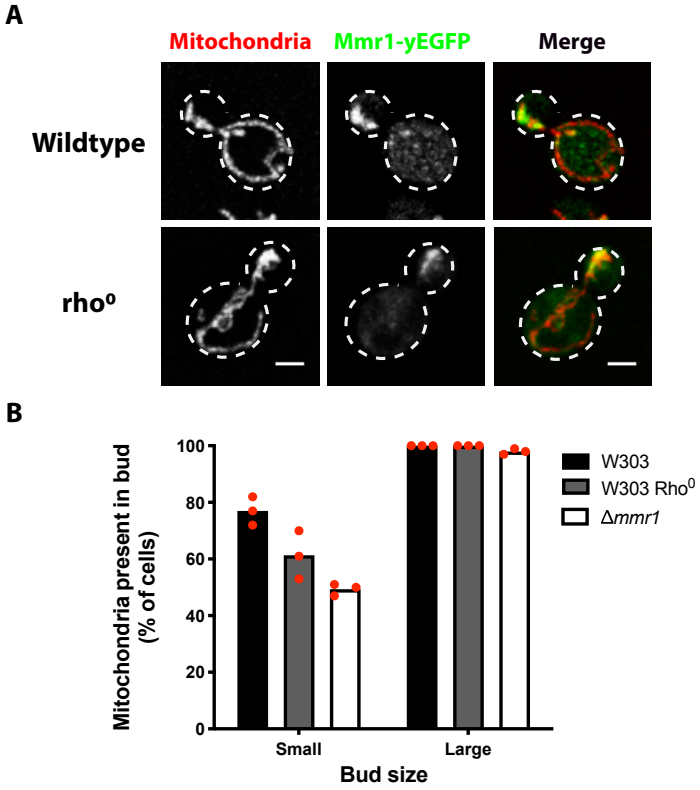

Figure 3.3: The lack of mtDNA affects proper mitochondrial inheritance. (A) Cells expressing mitoRED with Mmr1-yEGFP or rho^0 Mmr1-yEGFP, as indicated, were analyzed by fluorescence microscopy. (B) The presence of mitochondria in small and large buds was quantified in cells expressing wildtype, rho^0, and Δ*mmr1* along with mitoRED. Buds were classified based on the bud-to-mother-diameter ratio: small buds have a bud/mother-diameter ratio of <1/3 and large buds have a bud/mother-diameter ratio of ≥1/3.

3.E The function of Mmr1 in mitochondrial inheritance is reduced in respiration conditions

In respiration conditions, functional mtDNA is required for cell viability as the production of cellular energy is through the OXPHOS pathway. Given the differences between

Mmr1 and Ypt11 in the maintenance of mtDNA integrity, we examined the functions of Mmr1 and Ypt11 in cells grown in respiratory conditions. We first assessed the ability of Δ*mmr1* and Δ*ypt11* cells to inherit mitochondria. Interestingly, Δ*mmr1* cells show no mitochondrial inheritance delay in ethanol glycerol conditions, suggesting the function of Mmr1 in mitochondrial inheritance is reduced (Figure 3.4A). In contrast, Δ*ypt11* cells show only ~57% mitochondrial inheritance compare to ~77% mitochondrial inheritance in wildtype small buds. Interestingly, we noticed a qualitative difference in Mmr1 localization between cells grown in dextrose versus ethanol glycerol, consistent with the idea that Mmr1 function is altered in respiratory conditions (Figure callout). However, how Mmr1 is differentially regulated in respiration conditions remains unknown.

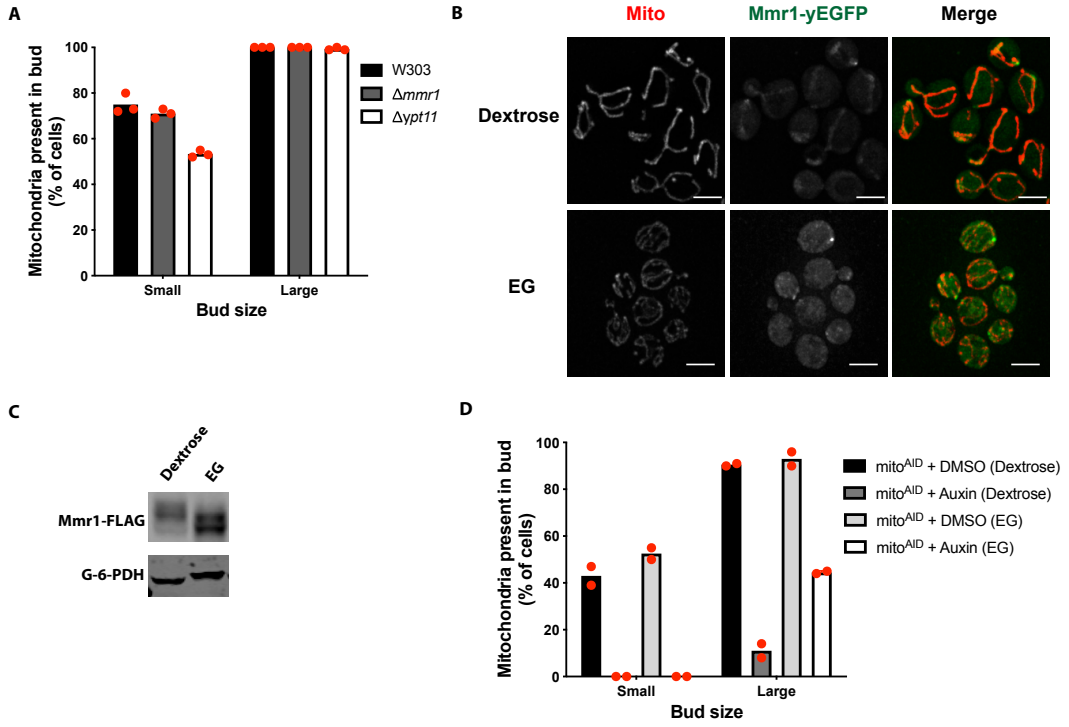

Figure 3.4: The function of Mmr1 in mitochondrial inheritance is reduced in respiration conditions. (A) The presence of mitochondria in small and large buds growing in ethanol glycerol (EG) was quantified in wildtype, Δ*ypt11*, and Δ*mmr1* cells expressing mitoRED. Buds were classified based on the bud-to-mother-diameter ratio: small buds have a bud/mother-diameter ratio of <1/3 and large buds have a bud/mother-diameter ratio of ≥1/3. (B) Cells expressing mitoRED with Mmr1-yEGFP in either dextrose or EG, as indicated, were analyzed by fluorescence microscopy. (C) Whole cell extracts of strains expressing Mmr1-FLAG from the endogenous *MMR1* promoter in either dextrose or ethanol glycerol

Figure 3.4 (continue from previous page)
growth conditions, as indicated, were analyzed by SDS-PAGE and western blot using anti-FLAG to detect the Mmr1-FLAG truncations and anti-G-6-PDH as a loading control. (D) The presence of mitochondria in small and large buds was quantified in *mitoAID* expressing mitoRED grown in dextrose with or without auxin, and mitoAID cells expressing mitoRED grown in EG with or without auxin. Buds were classified the same way as in A.

Growth in different carbon sources can alter protein expression levels and protein modifications. To assess Mmr1 modification in different carbon conditions, cells expressing endogenously FLAG tagged Mmr1 were grown in either ethanol glycerol or dextrose and western blot analysis was used to examine protein modification. We observed Mmr1-FLAG ran as a triplet, suggesting different species of Mmr1 are present in cells (Figure 3.4B). The triplet-banding patterns are different in dextrose and ethanol glycerol, suggesting the change in Mmr1 modification could be a potential mechanism used to regulate Mmr1 in respiration. In respiration conditions, the reduction in the function of Mmr1 leaves Ypt11 as the only other known adaptor to facilitate mitochondrial transport. However, in Δ*ypt11* cells, ~50% of small budded cells still inherited mitochondria, suggesting that a Ypt11- and Mmr1-independent mechanism of mitochondrial inheritance in respiration conditions. When conditionally depleting Mmr1 in Δ*ypt11* cells grown in ethanol glycerol, mitochondrial inheritance in small budded cells is nearly abolished; however, ~45% of large buds contained mitochondria, which is in marked contrast to the ~10% of large buds observed to have mitochondria when Mmr1 was depleted in Δ*ypt11* cells grown in dextrose (Figure 3.4C). Together, these data suggest an additional mechanism for mitochondrial inheritance in respiration conditions.

3.F Conclusions and Discussion

Here we provide data showing a potential role for Mmr1 in the maintenance of mtDNA. Mmr1 is important for maintaining functional mtDNA over generations as the deletion of *MMR1*

led to an increase in the number of petite colonies in a growing culture. The petite colonies were a mixed population: the majority of petite cells had non-functional mtDNA, while a smaller fraction lacked mtDNA. At this point, the reason Δ*mmr1* cells produce a mixed population of petite cells is unclear. In the future, we will examine the evolution of the petite phenotype over time to determine if Δ*mmr1* cells first become rho^{-} before becoming rho^{0}.

We observed that cells lacking mtDNA exhibit a slight delay in mitochondrial inheritance. Whether mtDNA physically associates with or indirectly impacts mitochondrial inheritance still remains unknown. One hypothesis is that Mmr1 interacts with functional mtDNA via physical interactions of inner and outer mitochondrial membrane proteins. This allows the mtDNA to be linked to mitochondrial transport motors to facilitate mtDNA inheritance into the daughter cell. The second hypothesis is that Mmr1 directly interacts with higher functioning mitochondria. Given that higher functioning mitochondria are linked to functional mtDNA, this indirectly selects for the inheritance of mtDNA. Research in the *Drosophila* female germline has shown that mitochondria containing mutated genomes produce less ATP and are selected against by degradation, leaving mitochondria with wildtype mtDNA behind. This may suggest low levels of ATP act as a signal for low functioning mitochondria, which the cell selects against. This is supported by the fact that wildtype and mutant mitochondria are both selected against when ATP levels are experimentally reduced. Hence, non-functional mtDNA produces less ATP, triggering the degradation of mitochondria (Lieber *et al*., 2019).

When examining if the mitochondrial transport role of Mmr1 is important to maintain functional mtDNA in a population, we found Mmr1 mutants that are defective in mitochondrial inheritance also lack the ability to maintain functional mtDNA similar to a Δ*mmr1* phenotype.

This finding suggests the mitochondrial transport role of Mmr1 is also important for maintaining mtDNA in a population. Studies have shown positive genetic interactions between *NUM1* and *MMR1*, and deletion of both genes rescues the unequal distribution of mitochondria between mother and bud observed in single deletions, restoring proper mitochondrial partitioning. Our preliminary data suggest that deletion of *NUM1* in Δ*mmr1* cells can partially restore the cells ability to maintain functional mtDNA in the population. The experiment conducted once showed Δ*mmr1*Δ*num1* cells have petite frequency of 42% while Δmmr1 cells have 65%This suggests that restoring mitochondrial partitioning at the compartment level does partially restore mtDNA partitioning; however, Mmr1 may play a more active role in transporting functional mtDNA into the bud.

Our data suggest that Mmr1 is differentially modified in different growth conditions to properly facilitate mitochondrial inheritance. Unlike cells growing in dextrose conditions, Δ*mmr1* cells growing in respiratory (ethanol glycerol) conditions do not have any mitochondrial inheritance delay suggesting the function of Mmr1 in mitochondrial inheritance is reduced. This leaves Ypt11 as the only known mitochondrial adaptor; however, deletion of *YPT11* does not completely abolish mitochondrial inheritance. When conditionally deleting Mmr1 in Δ*ypt11* cells, mitochondrial inheritance in small budded cells is nearly abolished; however, mitochondrial inheritance is observed in large budded cells, suggesting an additional mechanism for mitochondrial inheritance in respiratory conditions.

Together, we predict that in fermentation conditions, Mmr1 plays a role in the transport of functional mtDNA to the bud; however, in respiratory conditions, the amount of functional mtDNA is increased, therefore, Mmr1 is not needed for the selective transport of functional mitochondria into the bud.

Chapter 4:

Final conclusions and future directions

4.A Summary of findings

It is well known that many membrane-bound organelles cannot be generated de novo; therefore, the transport and tethering mechanisms required for their inheritance are critical for cell viability. This study utilizes the strengths of yeast as a model organism and a multidisciplinary approach, combining live-cell imaging and biochemical techniques, to elucidate the role of Mmr1 in mitochondrial and mtDNA inheritance.

In Chapter 2, we elucidated the domains of Mmr1 that govern its interactions with mitochondria, Myo2 and itself in order to provide insight into the molecular mechanism of Mmr1-dependent mitochondrial inheritance. We identified the region of Mmr1 that is both necessary and sufficient to interact with mitochondria. Specifically, we demonstrated that Mmr1 interacts directly with the mitochondrial membrane with a strong preference for cardiolipin, a mitochondria-specific phospholipid. We also identified the region of Mmr1 that is necessary and sufficient for self-interaction. Self-interaction enhances the interaction between Mmr1 and Myo2; it also likely enhances the interaction between Mmr1 and mitochondria by increasing the number of membrane binding sites per functional unit and, therefore, the avidity of Mmr1 for mitochondria.

In Chapter 3, we demonstrated that Mmr1, not Ypt11, plays a role in maintaining mitochondrial genome integrity. Specifically, both the Mmr1-mitochondria and Mmr1-Myo2 interactions are important for mitochondrial genome integrity. Conversely, lack of mtDNA negatively affects mitochondrial transport. These data suggest that mitochondrial inheritance is linked to the presence of functional mtDNA. Adding to the complexity of mitochondrial inheritance, preliminary data has suggested that in respiratory conditions, the role of Mmr1 in

mitochondrial inheritance is reduced. We hypothesize that the differential requirement for Mmr1 in fermentative and respiratory conditions is regulated by differential modification of Mmr1.

4.B Future directions and outstanding questions

The findings of this study raise many important and fascinating questions: 1. How are the functions of Mmr1 regulated? 2. What are the functions of Mmr1 post-mitochondrial transport? 3. How is the inheritance of mitochondria coupled with the inheritance mtDNA? 4. Is Mmr1 actively promoting the inheritance of mtDNA? If so, what is the mechanism?

4.C The order of operations of Mmr1-mitochondria and Mmr1-Myo2 interactions

In this study, I have shown Mmr1 interacts with itself, mitochondria, and Myo2 to properly transport mitochondria during cell division. However, the regulation and order by which Mmr1 interacts with its three binding partners remain unknown. Mmr1 self-interaction is important for the Mmr1-Myo2 interaction, suggesting that Mmr1 interacts with itself before interacting with Myo2 or that the self-interaction increases the avidity of the Mmr1-Myo2 interaction. Additionally, in the absence of the Mmr1 mitoBD, Mmr1 can still interact with Myo2, suggesting its interaction with Myo2 is independent of its ability to interact with mitochondria. On the other hand, in the absence of its Myo2 binding domain and/or the CC domain, Mmr1 can still interact with mitochondria, suggesting its interaction with mitochondria is independent of its ability to self-interact and interact with Myo2. Because Mmr1 can interact with both mitochondria and Myo2 independent of the other binding partner, the order in which Mmr1 interacts with Myo2, mitochondria and itself is still unknown. To better understand the order in which Mmr1 interacts with its binding partner, we could in vitro reconstitute the

interaction of Mmr1 with its binding partners by adding purified mitochondria, and Myo2 sequentially to Mmr1 and Mmr1ΔCC.

Furthermore, what regulates the interactions between Mmr1 and its binding partners remains unknown. The key to Mmr1 regulation maybe found in the highly phosphorylated nature of Mmr1. Our western blot analysis and the data from others have demonstrated that Mmr1 is differentially modified. We speculate this differential modification is important for the spatial and temporal regulation of the interactions between Mmr1 and its binding partners. To test this hypothesis, we could use phospho-null and phospho-mimetic mutants to test the effects of the PTMs on the interactions in cells and in vitro. A high throughput screen indicates that one of the 11 putative phosphorylation sites is phosphorylated by Cdk1, through a physical interaction with the kinase (Albuquerque *et al*., 2008). Other studies have hinted that other phosphorylation sites may also be phosphorylated with Cdk1 (Peng *et al*., 2008). We hypothesize Cdk1 plays an essential role in the regulation of Mmr1 function. To test whether Cdk1 directly plays a role in the regulation of Mmr1, I will test the effect of an ATP analog sensitive *cdc28* mutant on Mmr1 phosphorylation states.

4.D The interaction between Mmr1 and Mdm36

We have investigated additional interacting partners of Mmr1 through a yeast two-hybrid (Y2H) screen. These data revealed a novel interaction between Mmr1 and Mdm36, which is a component of the MECA complex and is required for normal mitochondrial morphology. Our preliminary data suggest Mmr1 loses its bud-specific localization in Δ*mdm36* cells. Mdm36 regulation of Mmr1 localization suggests a potential role in regulating Mmr1 function in the cells. When assessing the Mmr1-Mdm36 interaction *in vitro* using Mmr1 purified from *E. coli*

and Mmr1 purified from yeast, our preliminary data show Mmr1 purified from *E. coli* does not interact with Mdm36 but Mmr1 purified from yeast does. *E. coli* lacks the post-translational modification (PTM) machinery found in eukaryotic cells, thus, Mmr1 purified from *E. coli* is unmodified. The notion that yeast purified Mmr1 contains PTMs and *E. coli* purified Mmr1 does not is supported experimentally (shown in appendix). We reason that the lack of PTMs could be affecting the Mmr1-Mdm36 interaction; therefore, Mmr1 purified from yeast may be able to interact with Mdm36. Preliminary data obtained using Mmr1 purified from yeast are consistent with the possibility that PTMs in Mmr1 are essential for the interaction. This suggests that Mmr1 PTMs are involved the Mmr1-Mdm36 interaction and, consequently, the regulation of Mmr1 by Mdm36. It is possible that the PTMs of Mdm36 may also affect its interaction with Mmr1. We could explore these possibilities by purifying the proteins and phosphorylation site mutants from yeast and assessing their ability to interact pre- and post-phosphatase treatment.

4.E The potential crosstalk between Mmr1 and Num1

The functional significance and the molecular basis of the Mmr1-Mdm36 interaction remains unknown. Data published from our lab on the MECA complex show Mdm36 directly interacts with Num1 to facilitate the assembly and robustness of tethering of the MECA complex (Ping *et al.,* 2016). A role for Mdm36 in both Mmr1 and MECA function suggesting Mdm36 may be important to facilitate crosstalk between Mmr1 and Num1. Mmr1 and Num1 are both mitochondrial tethers, and they play an antagonist role in the cell. Mmr1 transports mitochondria to the bud and retains mitochondria at the bud tip, while Num1 retains mitochondria in the mother cell. Our work demonstrates that mitochondria drive the assembly of Num1 clusters, and Num1 begins to assemble and function in large buds. Once a Num1 cluster is formed; it tethers

mitochondria for the duration of the cell cycle. Prior to Num1 formation, Mmr1 is suggested to tether mitochondria. The timing of these events suggests a possible handoff from Mmr1 to Num1 and one candidate that would facilitate this handoff is Mdm36. Consistent with this hypothesis, both Mmr1 and Num1 preferentially interact with CL *in vitro* suggesting they may interact with a similar binding site on mitochondrial membrane that is enriched in CL.

Num1 still clusters and tethers mitochondria in the absence of Mmr1, suggesting Num1 function is independent of Mmr1. Work from our advisor has shown that deletion of *MDM36* results in smaller Num1 clusters (Lackner *et al*., 2013). Therefore, Mdm36 functions in facilitating the assembly of Num1. We speculate that Mdm36 may have a similar function in facilitating Mmr1 assembly. How Mdm36 interacts with both Mmr1 and Num1 is unknown. Whether Mmr1 and Num1 compete for the same pool of Mdm36 or if Mdm36 is present in different pools that are differently modified for interaction with either Mmr1 or Num1 is also unknown. We could overexpress either Mmr1 or Num1 to see if the overexpression of one protein would alter the localization and function of the other protein to investigate whether Mmr1 and Num1 are competing for the same pool of Mdm36. Once we assess the Mmr1-Mdm36 interaction *in vitro*, we could form the Num1-Mdm36 complex and add Mmr1 to see if Mmr1 competes with Num1 for Mdm36.

4.F The function of Mmr1 post-mitochondrial transport

This study mainly focuses on the transport role of Mmr1. However, the role of Mmr1 post-transport is still unknown. Studies have suggested that Mmr1 functions post-mitochondrial transport to tether mitochondria to cER at the bud tip (Swayne *et al*., 2011). However, the evidence is mostly indirect. Whether Mmr1 functions to tether mitochondria at the bud tip and

what Mmr1 associates with at the bud tip remain unclear. Myo2 has been shown to localize to the site of bud emergence in unbudded cells and to the bud tip in small buds. In large buds, Myo2 is localized at the bud neck (Lillie *et al*., 1994). Most organelle adaptors are released from Myo2 in order to properly position organelles in the bud. A mutant of the vacuole adaptor, Vac17, that is unable to be released from Myo2 transports vacuoles back to the bud neck (Yau *et al*., 2014, Tang *et al*., 2003), causing a defect in vacuole positioning. Based on our imaging of Mmr1-yEGFP localization, we speculate Mmr1 is released from Myo2 because I do not observe Mmr1 localizing to the bud neck. In order to assess the function of Mmr1 downstream of mitochondrial transport and provide evidence for the release of Mmr1 from Myo2, we will have to perform long-term imaging to visualize the localization of both Mmr1 and Myo2 throughout the cell cycle. This would provide stronger evidence that post-mitochondrial transport, Mmr1 shifts its role from transporting mitochondria to tethering mitochondria. We could also make mutants that artificially link Mmr1 to Myo2 and compare their post transport localization to the localization of wildtype Mmr1.

4.G Functional differences between Mmr1 and Ypt11

Work from our lab and others have demonstrated in fermentation (dextrose) conditions, Mmr1 and Ypt11 facilitate the transport of mitochondria into the growing bud. Although both adaptors have been shown to potentially tether mitochondria to the bud tip, the data supporting Ypt11 are speculative (Boldogh *et al*., 2004, Vevea *et al*., 2015). In respiration (ethanol glycerol) conditions, the function of Mmr1, not Ypt11, in mitochondrial inheritance is reduced. This suggests that in respiration conditions Ypt11 may play a larger role in mitochondrial inheritance. Deletion of *YPT11* does not abolish all mitochondrial inheritance suggesting the presence of

another mitochondrial inheritance mechanism. These findings give rise to many questions: 1. How is Mmr1 differentially regulated in these different carbon conditions? 2. Why is the function of Mmr1 reduced in respiratory conditions? 3. If a different mitochondrial inheritance mechanism exists, what would that be? It would be interesting to look into how Mmr1 modifications may contribute to this regulation of Mmr1. In addition, we can probe the physical interaction network of Mmr1 using IP mass spec and/or TurboID in cells grown in both dextrose and ethanol glycerol. This would further dissect the functions of Mmr1 in these growth conditions.

4.H Additional functions of Mmr1

In asymmetric cell division, inheriting the right quantity and the right quality of mitochondria are equally important. How cells determine the 'fitness' of mitochondria and partition them into two distinct cells remains largely unknown. Mmr1 plays an important role in anchoring high-functioning mitochondria to the tips of the daughter cells (McFaline-Figueroa *et al*., 2011; Pernice *et al*., 2016).

Our study shows Mmr1 might function in the maintenance of mitochondrial genome integrity. This role differentiates Mmr1 from its partially redundant adaptor Ypt11. Whether Mmr1 functions to actively transport functional mtDNA into the daughter cells or to maintain functional mtDNA is unclear. However, the role in maintaining mitochondrial genome integrity requires that Mmr1 interacts with mitochondria and Myo2, suggesting Mmr1 likely functions by facilitating the transport of functional mtDNA. To assess whether the role of Mmr1 in mitochondrial transport is important for the transport of mtDNA, we can track nucleoid inheritance over time using live cell imaging. We are able to visualize distinct nucleoids with a

fluorescent protein-tagged nucleoid-associated protein, Rim1. Specifically, in wildtype cells, and Mmr1 and Ypt11 mutants, we could examine 1) the percent of mitochondrial invading tips containing nucleoids to assess if Mmr1 has a preference in transporting mitochondrial tubules containing nucleoids, 2) the number of nucleoids in relationship to bud size to determine if nucleoid inheritance is altered, and 3) the localization of Mmr1 with respect to nucleoids. In addition, we could follow up with Mmr1 co-IPs to identify any potential candidates that can facilitate the Mmr1-mtDNA interaction.

4.I Additional transport mechanism in respiration conditions

This study has shown that Δ*mmr1* cells growing in respiration conditions do not have any mitochondrial inheritance delay suggesting the function of Mmr1 in mitochondrial inheritance is reduced. When conditionally depleting Mmr1 in a Δ*ypt11* background, mitochondrial inheritance in small budded cells is completely abolished; however, mitochondrial inheritance in large budded cells is increased by ~45% compared to ~10% in the wildtype. This suggests an additional mechanism for mitochondrial inheritance in respiration conditions. One possible candidate is a mitochondrial rho (Miro) GTPase, Gem1. Gem1 is a potential regulatory subunit of the ER-mitochondria encounter structure (ERMES) and functions in regulating the number and size of the ERMES complexes (Kornmann *et al*., 2011). Research has shown Gem1 along with Mmr1 and Ypt11 are important for mitochondrial inheritance in the W303 strain background. In the absence of *GEM1*, cells contain globular mitochondria and display an inheritance delay phenotype in small budded cells. In the W303 background, Δ*mmr1*Δ*ypt11* cells are severely sick and the additional deletion of *GEM1* results in even sicker cells with a doubling time greater than 12 hours (Frederick *et al*., 2008). These cells lack visible

mitochondria suggesting Gem1 plays a role in mitochondrial inheritance that is independent of Mmr1 and Ypt11. One hypothesis for Gem1's role in mitochondrial inheritance is through its function in the ERMES complex where ERMES creates contact between mitochondria and the ER. Through this contact, mitochondria may be inherited via hitchhiking on the ER.

We hypothesize that Gem1 may play a more prominent role in facilitating mitochondrial inheritance in respiratory conditions. In respiratory conditions, the volume of mitochondria increases and the contact between mitochondria and ER also increases; hence, indirectly more mitochondria may be inherited into the bud. In order to test this hypothesis, we would conduct long-term imaging of the ERMES complex during cell division and observe its inheritance with the inheritance of mitochondria. In addition, we could use conditional ERMES mutations with the vacuolar protein sorting 13, Vps13, mutant that bypasses the ERMES phenotype to assess if the inheritance of mitochondria is further reduced in Δ*ypt11* or Mmr1^{AID} Δ*ypt11* in the absence of the mitochondria-ER contact site.

4.J Final remarks

It is clear that mitochondria and mtDNA are critical for the health of virtually all eukaryotic cells. However, many questions regarding the inheritance of mitochondria and mtDNA remain unanswered. In the past decade, researchers have provided basic insight into these processes, which has opened the door to more mechanistic questions that remain to be answered. The field is actively searching for the mechanism involved in the asymmetric partitioning of mitochondria based on function in asymmetrically dividing cells. Questions that are actively being pursued are 1) Is mtDNA being differentially partitioned between cells? 2) Does improper mtDNA partitioning directly affect mitochondrial function and consequently the

asymmetry of the cell? 3) How are mtDNA and functional mitochondria being selected for transport into the daughter cells? The work presented here provides insights into the mechanism of mitochondrial inheritance that can be used to address these relevant and outstanding questions.

CHAPTER 5:
Materials and Methods

Cell growth prior to imaging

For all imaging, cells were grown to log phase in the appreciate selective synthetic media at 24°C unless otherwise indicated below. Special imaging conditions are as follows:

For Figure 2.6D: cultures were grown in the presence of 0.5 nM estradiol and 0.05 nM estradiol were added to the GalS::Mmr1-yEGFP and GalS::Mmr1ΔCC-yEGFP cultures, respectively, 5 h before imaging.

For Figure 3.2A: Cultures were grown in the presence of 1 ng/uL of DAPI for 30 mins prior to imaging.

Imaging acquisition and processing

For all imaging, cells were grown as described above at 24°C, concentrated by centrifugation, and mounted on a 4% wt/vol agarose pad. All imaging was performed at 22°C. Z series of cells were imaged at a single time point using a spinning disk confocal system (Leica) fit with a spinning disk head (CSU-X1; Yokogawa), a PLAN APO 100× 1.44 NA objective (Leica), and an electron-multiplying charge-coupled device camera (Evolve 512 Delta; Photometrics). A step size of 0.4 μm was used. Image capture was done using Metamorph (Molecular Devices). The images were deconvolved using AutoQuant X3's (Media Cybernetics) iterative, constrained 3D deconvolution method. Fiji (National Institutes of Health) and Photoshop (Adobe) were used to make linear adjustments to brightness and contrast. Deconvolved images are shown.

Image Analysis and Quantification

For the quantification of mitochondrial inheritance and the polarized localization of yEGFP fusion proteins in small buds, buds were classified as follows: small buds have a bud/mother-diameter ratio of <1:3, and large buds have a bud/mother-diameter ratio of ≥1:3. For mitochondria to be scored as properly inherited, mitochondria need to cross the mother–bud neck. Measurements of bud size were done using Fiji.

Protein Purification

Mmr1, Mmr1^{4E}, and Mmr1(61-195) were purified from *E. coli* as follows. Starter cultures of BL21(λDE3)/RIPL cells harboring plasmids pET22b His6-T7-Mmr1, pET22b His6-T7-Mmr1^{4E}, or pWaldo Mmr1(61-195)-GFP-His8, from which the expression of the genes is driven by the T7 promoter**,** were grown overnight in Luria-Bertani (LB) medium with chloramphenicol (25 μg/ml), glucose (0.04%), and ampicillin (150 μg/ml) for pET22b or Kanamycin (50 μg/ml) for the Waldo vector. The starter cultures were used to inoculate 2 L of LB medium containing the same additions described above. The cells were grown at 37°C until an OD_{600} of 0.5 was reached. To induce protein expression, IPTG was added to a final concentration of 250 μM, and the cultures were grown for 2 hours at 30°C for His6-T7-Mmr1 and His6-T7-Mmr1^{4E} and 16 hours at 18°C for Mmr1(61-195)-GFP-His8. The cells were then harvested by velocity centrifugation at 5,000 x *g* for 15 min. The resulting pellet was resuspended in 1/200 volume of resuspension buffer (RB; 20 mM Hepes pH 7.0, 500 mM NaCl, 1.89 mM BME) +1X Protease Inhibitor Cocktail Set 1 (PIC; Millipore), quickly frozen in liquid N_2, and stored at -80°C. The cell suspension was quickly thawed in a room temperature water bath, PIC was added to 1X, and the thawed cell suspension was subjected to two more freeze-thaw cycles. The homogenate was sonicated briefly to further lyse cells and clarified by centrifugation at 17,000 x *g* for 45 minutes

at 4°C. The proteins were purified from the supernatant using HisPur Ni-NTA resin (Thermo Scientific). The supernatant was incubated with resin for 1 hour at 4°C, and the resin was then pelleted at 3,000 x *g* for 3 minutes. The protein bound resin was washed three times with RB+1X PIC and three times with wash buffer (RB + 30 mM imidazole + 0.25X PIC) and was then loaded into a chromatography column. Protein was eluted from the column using a step gradient of RB + 60-300 mM imidazole. 5 μL of each elution was mixed with sample buffer, run on a SDS-PAGE gel, and Coomassie stained. Mmr1, Mmr1^{4E}, or Mmr1(61-195) elutions were pooled and dialyzed overnight in 20 mM Hepes pH 7.0, 500 mM NaCl. Glycerol was added to 10%, and the protein was aliquoted, frozen in liquid N_2, and stored at -80°C. The concentration of the purified proteins was determined using a BCA protein assay kit (Pierce).

Liposome Floatation Assays

The following phospholipids were supplied in chloroform at 10 mg/ml from Avanti Polar Lipids, Inc: 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphate (PA), palmitoyl-oleoyl phosphatidylcholine (PC), palmitoyl-oleoyl phosphatidylethanolamine (PE), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (PG), soybean phosphatidylinositol (PI), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-(Phosphor-L-Serine) (PS), and tetraoleoyl-cardiolipin (CL). For OMC liposomes, individual phospholipids were mixed to achieve a mol% composition that mimics the mitochondrial outer membrane: 46% PC, 33% PE, 10% PI, 4% PA, 1% PS, 6% CL (Zinser and Daum, 1995). For OMC+0% CL and OMC+17% CL, compensatory changes were made in the percentage of PC present in the lipid mixture. To examine lipid specificity, 20 or 40 mol% of the indicated phospholipid was mixed with 80 or 60 mol% PC, respectively. Headgroup-labeled

lissamine rhodamine B phosphatidylethanolamine (Rd-PE) was added to all liposome mixtures in trace amounts.

Lipid mixtures were placed in a vacuum chamber overnight. The lipid films were rehydrated with 20 mM Hepes pH 7.0 to a final lipid concentration of 2 mg/ml at room temperature for 1 hour. Lipid mixtures were pipetted up and down to create a heterogeneous population of liposomes. Purified proteins and liposomes, as indicated, were added to gradient reaction buffer (GRB; 20 mM Hepes, pH 7.0 and 150 mM NaCl) for a total volume of 100 μl. This reaction was left at room temperature for 20 min. 400 μl 50% sucrose in GRB was added to the reaction mixture and added to the bottom of a 13 x 51 mm polycarbonate centrifuge tube (Beckman). The reaction plus sucrose mixture was overlaid with 1 ml 30% sucrose in GRB, 500 μl 10% sucrose in GRB, and 250 μl 0% sucrose in GRB for a total volume of 2.5 ml. Sucrose gradients were subjected to centrifugation in a Beckman SW55 rotor at 200,000 x g at 4°C for 2 hours. Two 1.25 mL fractions were pipetted from the top, resulting in a top and bottom fraction. To monitor the efficiency of the liposome floats, the rhodamine fluorescence of each fraction was quantified using a SpectraMax M5 plate reader (Molecular Devices) with the excitation and emission monochromators set at 550 nm and 590 nm, respectively. In all cases, >85% of liposomes were observed in the top fraction. To quantify the fraction of protein that floated with the liposomes, equal volumes of top and bottom fractions were analyzed by SDS-PAGE followed by western analysis using mouse monoclonal anti-T7 or anti-GFP (Invitrogen) for the primary antibody and goat anti-mouse IgG Dylight 680 (Pierce) or goat anti-rabbit IgG DyLight 800 (Thermo Fisher Scientific), respectively, for the secondary antibody. The immunoreactive bands were detected with the Odyssey Infrared Imaging System (Li-Cor Biosciences) and quantified using the accompanying software (Image Studio). For the high salt floats, the lipids

were resuspended in 20 mM Hepes pH 7.0, 450 mM NaCl and the salt in the GRB was increased to 450 mM NaCl.

Cell extracts and western blots

The indicated strains were grown to midlog phase in YPD media. For Fig. 2.7 and C, 0.5 nM estradiol and 0.05 nM estradiol were added, as indicated, to drive expression of GalS::*MMR1-yEGFP* and GalS::*MMR1*Δ*CC-yEGFP*, respectively. 1.0 OD of cells were harvested, and whole cell extracts were prepared using a NaOH lysis and TCA precipitation procedure. Each TCA pellet was resuspended in 50 μl MURB (100 mM MES, pH 7, 1% SDS, and 3 M urea). Whole cell extracts were analyzed by SDS-PAGE followed by western analysis using anti-GFP (Invitrogen), anti-glucose-6-phosphate dehydrogenase (G-6-PDH; Sigma-Aldrich), or anti-phosphoglycerate kinase (PGK; Life Technologies) as the primary antibodies and goat anti-rabbit IgG DyLight 800 or goat anti-mouse IgG DyLight 680 (Thermo Fisher Scientific) as the secondary antibodies. The immunoreactive bands were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Immunoprecipitations

The indicated strains were grown to midlog log phase (~0.8 OD_{600}) in 50 ml YPD media. Cells were harvested, immediately frozen in liquid nitrogen, and stored at -80°C. Cell pellets were resuspended in 300 μl IP lysis buffer (IPLB; 20 mM Hepes KOH, pH 7.4, 150 mM KOAc, 2 mM $Mg(Ac)_2$, 1 mM EGTA, 0.6 M Sorbitol, Triton X-100) plus 1mM DTT, 1X Protease Inhibitor Cocktail Set 1 (Millipore), and phosphatase inhibitors (60 mM βglycerophosphate, 10 mM NaF, 1 mM sodium molybdate, 50 μM canthardin). Pre-chilled fine glass beads (0.5 mm

Glass Beads, BioSpec cat # 11079105) were added to the lysates until only ~5 mm of lysate remained above the beads. Lysates were vortexed seven times at a setting of 9 for 1-1.5 min at 4°C with 1-1.5 min rest on ice between each vortexing session. 0.1% Triton X was added to the lysates. Supernatants were removed from the glass beads by puncturing a hole in the bottom of each eppendorf tube using a syringe needle (23 gauge), placing the tubes over empty eppendorf tubes, and centrifuging the lysates into the new tubes at 0.9 x g for 30 sec. The lysates in the new tubes were centrifuged at 17,000 x g for 30 minutes at 4 °C to remove all large cell debris. 25 µl of anti-FLAG or anti-GFP µMACS beads (Miltenyi) were added to the supernatant, and the samples were placed on ice for 30 min. µMACS columns placed in magnetic holders were equilibrated with 250 µl IPLB + 0.1% Triton X-100 + PIC. The lysates were added to the equilibrated columns. Columns were washed with 800 µl IPLB + 0.1% Triton X-100 + PIC three times and with 500 µl IPLB, no detergent, no PIC twice. 25 µl 1X MURB were added to the columns and incubated for 10 min at room temperature. An additional 25 µl 1X MURB were added to the column, and the 50 µl elution volume was collected. The cell lysate (5 μl) and immunoprecipitation elution fractions (15 µl) were analyzed by SDS-PAGE followed by western analysis using anti-GFP (Invitrogen), anti-Myc (clone 9E10), or anti-FLAG (Sigma) as the primary antibodies and goat anti-rabbit IgG DyLight 800 or goat anti-mouse IgG DyLight 680 (Thermo Fisher Scientific) as the secondary antibodies. The immunoreactive bands were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences) and quantified using the accompanying software (Image Studio).

Yeast Two-Hybrid Analysis

PJ69-4A Δ*mmr1*::*NATNT2* and PJ69-4Alpha Δ*mmr1*::*NATNT2* were transformed with the indicated Gal4AD and Gal4BD fusions, respectively. PJ69-4A Δ*mmr1*::*NATNT2* cells harboring the indicated Gal4AD fusions were then mated with PJ69-4Alpha Δ*mmr1*::*NATNT2* cells harboring the indicated Gal4BD fusions. Diploids were selected by on SC–LEU–TRP+DEX plates, and protein–protein interactions were assessed by growth on SC–LEU–TRP–ADE+DEX plates at 24°C.

Petite frequency Assay

Strains were taken from agar plates with a non-fermentable carbon source (Ethanol Glycerol) and grown in YP-Ethanol Glycerol (YPEG) media at 30°C overnight. Overnight cultures were diluted to 0.15 OD in YPEG media in the morning, grown to log phase and maintained at the log phase for 24 hours in YPEG at 30°C. All cells growing under this condition must contain functional mtDNA because functional mtDNA is essential for respiration in a non-fermentable carbon source. The rest of the experimental method varies depending on the specific assay:

Petite frequency assay (48hrs): The cultures were then diluted to 0.15 OD in media containing a fermentable carbon source (YPD), cells were grown to log phase and maintained at log phase for 48 hours. This removes the selection for mtDNA. After 48hrs in log, the cultures were diluted 1:10,000 and 200 µL were plated on low Ade YPD plates and grown at 30°C for ~3 days until single cells give rise to distinct colonies.

Time course petite frequency assay: For time point 0 hour, the cultures were immediately diluted 1:10,000 and 200 µL were plated on low Ade YPD plates. The remaining culture were

diluted to an appropriate OD that will maintain log phase in YPD for the next time point. The time points taken are 6, 9, 23, 30 hours. All the plates were grown at 30°C for ~3 days until single cells gave rise to distinct colonies. For strains containing the AID tag, 1 mM of Auxin or an equivalent amount of DMSO (control) is added to the YPEG cultures growing in log for 23.5 hours and allowed 30mins of additional growth to deplete the AID tagged protein of interest. Then the culture can be plated as time point 0.

Lambda phosphatase treatment and phos-tag analysis of Mmr1

0.5 μM yeast purified Mmr1 proteins were treated with 400 units of lambda phosphatase (P0753S NEB) in 1X NEBuffer for Protein MetalloPhosphatases (PMP) and 1X 10 mM $MnCl_2$. The reaction is incubated at 30°C for 30 minutes. The reaction was run on a self-made phos-tag gel using Phos-tagTM AAL-107 from FUJIFILM Wako Chemicals U.S.A. Corporation.

CHAPTER 6:
References

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Appendix 1:

The role of phosphorylation in Mmr1

Introduction:

In budding yeast, organelle inheritance is tightly linked to the regulation of organelle specific adaptors for transport motors. Many of these adaptors are cell cycle regulated in a manner that is dependent on post-translational modifications (PTMs), such as phosphorylation. Vacuolar adaptor, Vac17, is a phosphoprotein and its phosphorylation is coordinated with the cell cycle and regulates vacuole inheritance (Peng et al., 2009). Studies have shown that both mRNA and protein levels of Vac17 oscillate during the cell cycle and the timing of Vac17 phosphorylation is directly link to vacuole inheritance. Moreover, Vac17 is phosphorylated by Cyclin-dependent kinase, Cdk1, which is a master regulator of mitotic and meiotic cell cycles. This type of phosphorylation is very common for many yeast proteins whose phosphorylation is cell cycle-dependent. Supporting similar regulation for the mitochondrial adaptor, Mmr1, and the peroxisome adaptor, Inp2, both contain multiple putative Cdk1 sites.

Here we are examining the role for phosphorylation in Mmr1 regulation. Mmr1 is a 491 amino acid protein that contains a mitochondrial binding domain, coiled-coil region and a well-studied Myo2 binding region. The protein contains 11 putative phosphorylation sites, one of which, S37, has been shown to be phosphorylated by Cdk1, and 2 putative ubiquitination sites. Experimentally, we have shown that Mmr1 is a phosphoprotein and the phosphorylation of Mmr1 may be important for the spatial and temporal regulation of the interactions between Mmr1 and its binding partners affecting Mmr1's function in mitochondrial inheritance.

Mmr1 is a phosphoprotein

Mmr1 purified from yeast migrates as a doublet on SDS-PAGE gels suggesting Mmr1 has more than one modification state in vivo. In addition, when Mmr1 purified from yeast is run

on a Phos-Tag gel, which separates protein by its phosphorylation states, Mmr1 separates into multiple, distinct bands supporting the idea that Mmr1 is differentially modified. To further test whether these bands represent phosphorylated forms of Mmr1, phosphatase was used to strip away phosphorylation modifications on Mmr1. Phosphatase treated Mmr1 migrates as a single band on a phos-tag gel suggesting that these distinct bands are indeed phosphorylated forms of Mmr1 (Figure A1.1).

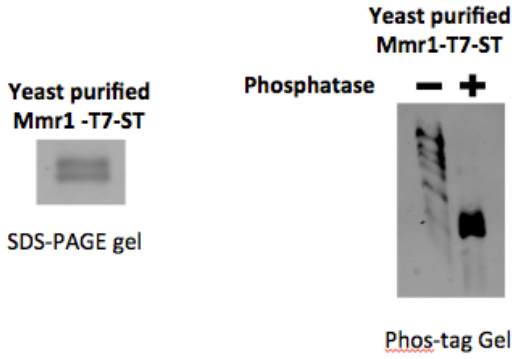

Figure A1.1: Mmr1 is a phosphoprotein. Yeast purified Mmr1-T7-ST was analyzed by SDS-PAGE and Western blot using anti-T7 to detect the Mmr1-T7-ST (A). Yeast purified Mmr1-T7-ST with and without lambda phosphatase treatment was analyzed by phos-tag Gel.

S81 and S83 are important for the Mmr1-mitochondria interaction

In Chapter 2, we showed that the mitochondrial binding region (mitoBD) of Mmr1 spans amino acids 76-195. The region containing 76-195aa of Mmr1 is necessary and sufficient to interact with mitochondria. Further truncation of the mitoBD by another 15 amino acids from the N-terminus results in the cytosolic localization of Mmr1 suggesting these 15 amino acids are critical for the Mmr1-mitochondrial interaction. Examining these 15 amino acids, we found two putative phosphorylation sites: S81 and S83. When substituting these two phosphorylation sites for Alanine to create a phospho-null mutant, mitoBD is still able to interact with mitochondria in vivo. However, when substituting these two phosphorylation sites with either Aspartic acid or Glutamic acid to create phospho-mimetic mutants, mitoBD no longer interacts with mitochondria

in vivo (Figure A1.2). These data suggest that the Mmr1-mitochondria interaction is regulated by phosphorylation. More specifically, phosphorylation functions to release Mmr1 from its interaction with mitochondria.

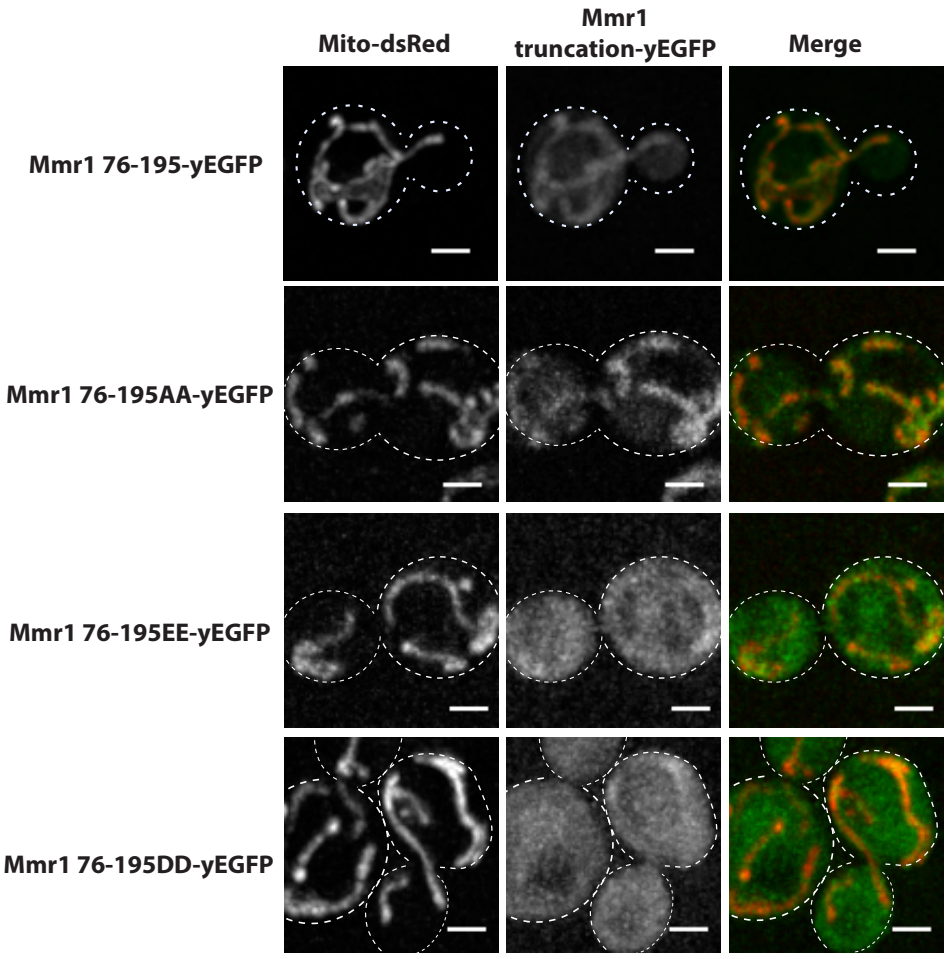

Figure A1.2: mitoBD with phosphor-mimetic mutations can no longer interact with mitochondria in cells. Cells expressing mitoRED and the indicated mitoBD mutations were analyzed by fluorescence microscopy. Whole cell projections are shown. The cell cortex is outlined with a dashed white line. Scale bar, 2 μm

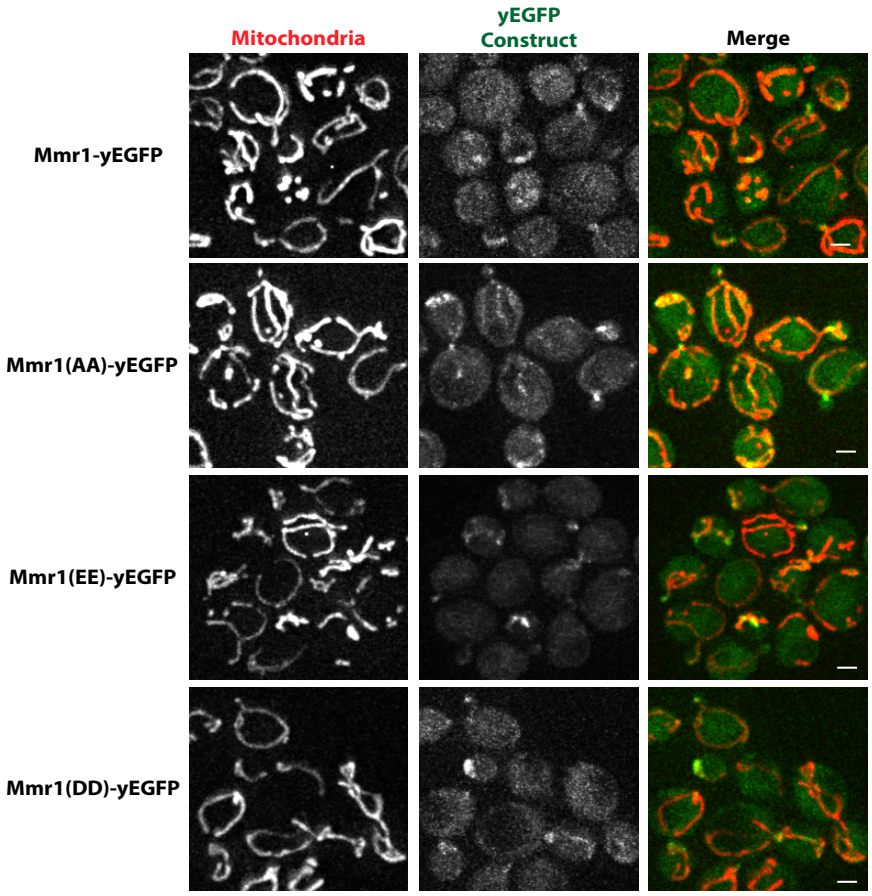

Figure A1.3: Full length Mmr1 with phosphor-mimetic mutations interact with mitochondria in cells similar to wildtype Mmr1. Cells expressing mitoRED and the indicated mutations in full length Mmr1 were analyzed by fluorescence microscopy. Whole cell projections are shown. The cell cortex is outlined with a dashed white line. Scale bar, 2 μm

Discussion:

Mmr1 purified from yeast indicates that Mmr1 is highly phosphorylated. The results with the mitoBD phospho-null and phospho-mimetic mutants suggest a role for Mmr1 phosphorylation at S81 and S83 is releasing Mmr1 from the mitochondrial membrane. Based on these data we propose the following potential model: 1) Mmr1 with non-phosphorylated S81 and S83 interacts with mitochondria allowing mitochondria to be trafficked into the bud. 2) Once at the bud tip, Mmr1 functions to tether mitochondria at the bud tip. 3) Mmr1 is then phosphorylated potentially by Cdk1. 4) The phosphorylation of Mmr1 results in its dissociation

from the mitochondrial membrane. The mother specific tether, MECA, is then able to function to tether mitochondria at the cell cortex (Figure A1.4).

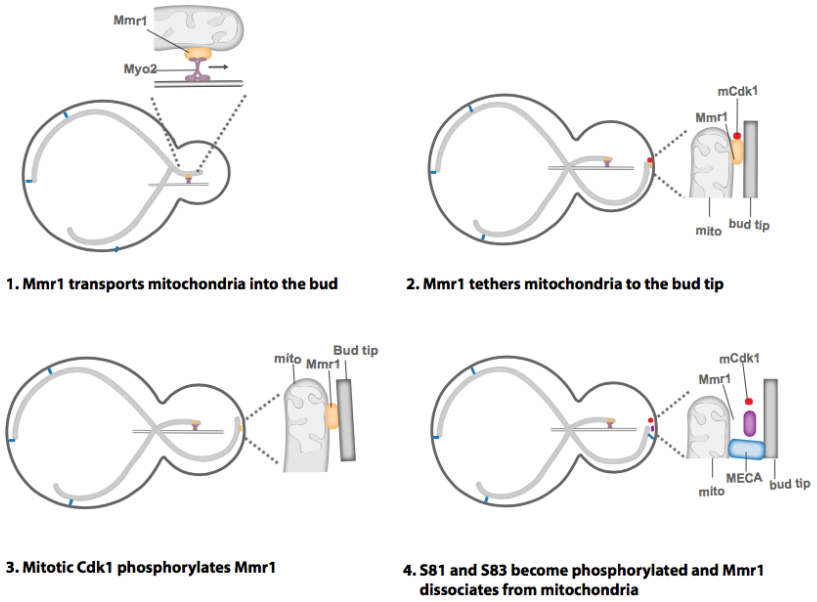

Figure A1.4: Proposed model. 1) Mmr1 with S81 and S83 not phosphorylated interacts with mitochondria and traffics mitochondria into the bud. 2) At the bud tip, Mmr1 functions to tether mitochondria. 3) Mmr1 get phosphorylated potentially by Cdk1 and 4) Then Mmr1 dissociates from the mitochondrial membrane and the mother specific tether, MECA, functions to tether mitochondria at the cell cortex.

However, the results obtained from the mitoBD phospho-null and phospho-mimetic mutants that support this model are complicated by the results observed when these mutations are made in full length Mmr1. When making S81 S83 phospho-null and phospho-mimetic in full length Mmr1, the phospho-null, Mmr1AA, remained bound to mitochondria as expected. However, surprisingly both the phospho-mimetics, Mmr1DD or Mmr1EE, also remained associated with mitochondria (Figure A1.3). This suggests that regulation of full length Mmr1 is more complex that the regulation of the mitoBD and suggests that additional phosphorylation sites may also be important for the Mmr1-mitochondria interaction.