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

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ABSTRACT Mitochondrial transport and anchoring mechanisms work in concert to position mitochondria to meet cellular needs. In yeast, Mmr1 functions as a mitochondrial adaptor for Myo2 to facilitate actin-based transport of mitochondria to the bud. Posttransport, Mmr1 is proposed to anchor mitochondria at the bud tip. Although both functions require an interaction between Mmr1 and mitochondria, the molecular basis of the Mmr1–mitochondria interaction 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.

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 Hollenberg, 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 (McFalone-Figueroa et al., 2011; Katajisto et al., 2015; Pernice et al., 2016, 2017; Kraft and Lackner, 2018). Although 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 is required for Myo2-dependent transport of mitochondria to buds (Itoh et al., 2002, 2004; Boldogh 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 endoplasmic reticulum (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.

RESULTS

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 whether 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 flotation 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 flotation assays, recombinant Mmr1 associated with OMC liposomes containing 6 and 17% CL, but not with OMC liposomes that lack CL (Figure 1A).

To further examine the specificity of the Mmr1–phospholipid interaction, we assessed the ability of Mmr1 to bind liposomes composed of the neutral phospholipid phosphatidyethanolamine (PE) plus one of the following phospholipids: CL, phosphatic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), or phosphatidylethanolamine (PE). When individual phospholipids were present at 20 mol%, Mmr1 associated only with CL-containing liposomes (Figure 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 flotation assays disrupted the Mmr1–phospholipid interaction, indicating that the interaction is electrostatic (Figure 1C). Together, these data indicate that Mmr1 can interact directly with phospholipid membranes in vitro and exhibits a preference for liposomes containing CL.

FIGURE 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.
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 2A). Western blot analysis confirmed that the proteins were expressed with minimal degradation (Figure 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 colocalized with mitochondria and exhibited a punctate, bud-enriched localization (Figure 2A; Itoh et al., 2004; Swayne et al., 2011; Eves et al., 2012). For the Mmr1 truncations, we found that Mmr1(61–195) colocalized with mitochondria, while the distribution of Mmr1(61–152) was shifted toward the cytosol. In addition, we found that Mmr1(76–195) colocalized with mitochondria but Mmr1(91–195) and Mmr1(76–152) were primarily cytosolic. In contrast to the polarized localization of wild-type Mmr1,
Mmr1(76–195) and the other mitochondrial-associated Mmr1 truncations appeared to be evenly distributed along mitochondria in the mother and bud (Figure 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 flotation 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 2C). Thus, our in vivo and in vitro studies indicate that amino acids 76–195 of Mmr1 compose the mitochondrial binding domain (mitoBD; Figure 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 colocalized with mitochondria (Figure 3A), consistent with the disruption of the Mmr1–mitochondria interaction. In addition, an enrichment of Mmr1Δ76–195-yEGFP in small buds was observed (Figure 3, A 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 (Supplemental Figure S1A). As discussed below, Mmr1Δ76–195 was also able to interact with itself in yeast two-hybrid assays (Figure 4B), 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 (Supplemental Figure S1B). Specifically, we constructed Mmr1 R80E R86E K95E K98E and will refer to this mutant as Mmr1 R80E R86E K95E K98E (Figure 3B). When expressed as a yEGFP fusion in cells, Mmr1 R80E R86E K95E K98E was able to interact with Myo2 but not itself, full-length Mmr1, or CC domain (Reed et al., 2012). Indeed, this construct was able to interact with Myo2 but not itself, full-length Mmr1, or Mmr1Δ76–195 (Figure 4, B and C). These results suggest that the CC domain is necessary and sufficient for the interaction with mitochondria, and the mitochondrial membrane is critical for Mmr1 function in mitochondrial inheritance.

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 2D; Itoh et al., 2004; Eves et al., 2012). Mmr1 also contains a predicted coiled-coil (CC) domain (Itoh et al., 2004). Although 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 (Itoh et al., 2004; Eves et al., 2012). To determine whether 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 Δmmr1 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 4A). 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 4A). 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 4, B 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 communoprecipitation assays. We coexpressed differentially tagged versions of Mmr1ΔCC in diploid cells and examined the ability of Mmr1ΔCC-FLAG to communoprecipitate Mmr1ΔCC-yEGFP. In comparison to the steady-state levels of wild-type Mmr1-FLAG and Mmr1-yEGFP, the steady-state levels of Mmr1ΔCC-FLAG and Mmr1ΔCC-yEGFP expressed from the endogenous MMR1 promoter were increased (Figure 4D; lysate). Despite the increased levels of the Mmr1ΔCC proteins, the ability of Mmr1ΔCC-FLAG to communoprecipitate Mmr1ΔCC-yEGFP was dramatically reduced compared with the ability of Mmr1-FLAG to communoprecipitate Mmr1-yEGFP (Figure 4, D and E). These results further support the idea that the CC domain of Mmr1 mediates Mmr1 self-interaction.

Direct membrane binding contributes to Mmr1 function in mitochondrial inheritance

We next assessed mitochondrial inheritance in cells expressing Mmr1Δ76–195-yEGFP and Mmr1Δ4E-yEGFP. In the absence of Mmr1, a greater fraction of small-budded cells are devoid of mitochondria in comparison to wild-type cells, indicative of a delay in the inheritance of mitochondria (Itoh et al., 2004). We found that cells expressing Mmr1Δ76–195-yEGFP and Mmr1Δ4E-yEGFP exhibit a delay in mitochondrial inheritance similar to that observed for cells lacking Mmr1 (Figure 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, mrr1ΔΔypt11 and mrr1ΔΔypt11 cells exhibited growth defects similar in severity to Δmmr1Δypt11 cells (Figure 3E). Together, these data indicate a direct interaction between Mmr1 and the mitochondrial membrane is critical for Mmr1 function in mitochondrial inheritance.
FIGURE 3: The membrane-binding domain of Mmr1 is required for Mmr1 function. (A, B) Cells expressing mitoRED and Mmr1-yEGFP, Mmr1Δ76–195-yEGFP, or Mmr1Δ4E-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 Mmr1Δ4E (5 µM) were incubated with OMC + 17% CL liposomes. The reactions were subjected to liposome flotation and analyzed as described in Figure 1A. Data are 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 Mmr1Δ4E-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) Δypt11Δmmr1, Δypt11 Δmmr1Δ76–195-yEGFP, and Δypt11 Δmmr1Δ4E-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.
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 5A). 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, nonsignificant defects in mitochondrial inheritance in otherwise wild-type and Δp11t1 backgrounds (Figure 5B), suggesting the function of Mmr1ΔCC in mitochondrial inheritance may be attenuated.

On the basis of our coimmunoprecipitation experiments, we noted that the levels of Mmr1ΔCC-yEGFP were higher than those of wild-type Mmr1-yEGFP. Indeed, the steady-state protein levels of Mmr1ΔCC-yEGFP were found to be ~16x that of Mmr1-yEGFP (Supplemental Figure S2, A and C). 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 similar to wild-type 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 Mmr1ΔCC-yEGFP and Mmr1ΔCC-yEGFP were comparable to that of Mmr1-yEGFP expressed from the endogenous MMR1 promoter (Figure 5C and Supplemental Figure S2, B and C). Cells expressing Mmr1-yEGFP from the estradiol-regulated GalS promoter inherited mitochondria similarly to cells expressing Mmr1ΔCC-yEGFP from the endogenous Mmr1 promoter (Figure 5, B 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 Δmmr1 cells (Figures 3D and 5D). These results indicate that, at wild-type 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.

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 (Supplemental Figure S2D). 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 (Figure 5, E and F), consistent with previous findings (Itoh et al., 2004). To test whether 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 5, E 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...
FIGURE 5: 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 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 3D (D). 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. 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.
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 S5F). These results suggest that, even though constructs lacking the CC domain can interact with Myo2 (Figure 4C; 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 Mmr1-CC and Myo2 in cells using communoprecipitation assays. We found that despite the increased levels of Mmr1-CC in cells, the ability of Mmr1-CC to communoprecipitate Myo2 was reduced compared with the ability of Mmr1 to communoprecipitate Myo2 (Figure 5, G 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.

**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 nonbilayer lipids, 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 binds 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. Although 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.

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.

**MATERIALS AND METHODS**

**Strains and plasmids**

Strain W303 (ade2–1; leu2–3; his3–11, 15; trp1–1; ura3–1; can1–100; Naylor et al., 2006) and the yeast two-hybrid strains PJ69-4A and PJ69-4alpha (MATa/alpha trp-1-901 leu2-3,112 ura3-52 his3-200 gal4αgal80 lys2::GAL1-HIS3 gal2-ade2 met2::GAL7-lacZ (gifts from S. Fields, University of Washington, and the Yeast Resource Center; James et al., 1996) were described previously. Tables of the strains, plasmids, and primers used in this study can be found in the Supplemental Material.

The following W303, PJ69-4A, and PJ69-4alpha gene deletion strains were obtained by replacing the complete ORF of the genes with the indicated cassette using PCR-based targeted homologous recombination: W303 Δppt1::NATNT2, W303 Δmnr1::KANMX6, W303 Δacr1::KANMX6, W303 Δclcd1::KANMX6, W303 Δata1::NATNT2, P69-4A Δmnr1::NATNT2, and P69-4alpha Δmnr1::NATNT2 (Longtine et al., 1998; Janke et al., 2004). The functional C-terminally tagged strains MMR1-yEGFP::His (Mmr1–yEGFP), MMR1-FLAG::His (Mmr1–FLAG), and Myo2-Myc::His were constructed by PCR-based targeted homologous recombination using pFA6a-link-yEGFP::SpHi55 (∝KXT128), pFA6a-FLAG-His3MX6, and pFA6a-13Myc-His3MX6 (Sheff and Thorn, 2004; Hoppins et al., 2011).
MMR1(Δ76–195)-yEGFP::His, MMR1Δ288–377)-yEGFP::His, MMR1Δ288–377)-yEGFP::His (referred to as Mmr1ΔCC-yEGFP), MMR1Δ288–377)-FLAG::His (referred to as Mmr1ΔACC-FLAG), and MMR1Δ4E-yEGFP::His were constructed by PCR-based targeted homologous recombination in which three PCR products were transformed into Δmmr1::KANMX6. PCR product #1 encoded the MMR1 coding sequence upstream of the deletion with homology to the genome downstream of the MMR1 locus at the 5’ end and homology to the yEGFP cassette at the 3’ end. PCR product #2 encoded the yEGFP::SphiHis cassette with homology to the end of the MMR1 truncation at the 5’ end and homology to the genome downstream from the MMR1 locus at the 3’ end. For GCN4CC-Mmr1(378–491)-yEGFP::His, a gBlock containing the GCN4CC-Mmr1(378–491) sequence was used as the template for PCR product #1. The GCN4 CC sequences encodes amino acids 249–281 of GCN4 and is fused to Mmr1(378–491) via a GA linker (O’Shea et al., 1991).

To regulate MMR1-yEGFP and MMR1ΔCC-yEGFP expression using estradiol, the Gal8p promoter was placed upstream of the MMR1 coding sequence in the genome. The Gal8p::MMR1-yEGFP::NAT::His and Gal8p::MMR1ΔCC-yEGFP::NAT::His strains were constructed by PCR-based targeted homologous recombination using pYM-N31 (Janke et al., 2004) and the MMR1-yEGFP::His and MMR1ΔCC-yEGFP::His strains described above. The strains were then transformed with Mss1-linearized pAGL. pAGL encodes for GAL4-EstrogenBD-VP16::NATMX6, which is the transcription factor used for estradiol control of GAL promoters (Veatch et al., 2009).

Haploid double-mutant/tagged strains were generated by mating, followed by sporulation and tetrad analysis or by PCR-based targeted homologous recombination.

The plasmids pXY142-mitodsRED (mitoRED; Friedman et al., 2011), pAGL (Veatch et al., 2009), pET22b His6-T7 (Ping et al., 2016), pWaldo-GFPd (a gift from Heather Pinkett, Northwestern University; Drew et al., 2006), pGADCl1-Myc2 cargo-binding domain (CBD; Pashkova et al., 2005), and pGBK7-BamHI and pGAD77-BamHI (Lackner et al., 2013) were described previously.

pET22b His6-T7 and pWaldo-GFPd modMCS were used to over-express proteins in E. coli. To construct pET22b His6-T7-Mmr1 and pET22b His6-T7-Mmr1Δ4E, Mmr1 and Mmr1Δ4E were PCR amplified from genomic DNA isolated from W303 and W303 MMR1Δ4E-yEGFP, digested with BglII and Xhol, and cloned into pET22b His6-T7, which was digested with BamHI and Xhol. To construct pWaldo Mmr1(Δ61–195)-GFP, pWaldo-GFPd modMCS was first constructed by modifying the multiple cloning site to 5’-CATATGGGATCCAC-

Imaging

For Figures 2, 3, and 5, the indicated cells harboring mitoRED were grown to mid-log phase in SC-LEU + 2% (wt/vol) dextrose media with 2x adenine. For Figure 5D, 0.5 nM estradiol and 0.05 nM estradiol were added to the Gal8p::Mmr1-yEGFP and Gal8p::Mmr1ΔCC-yEGFP cultures, respectively, 5 h before imaging.

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 100x 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. Deconvoluted images are shown.

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, Mmr1Δ4E, and Mmr1(Δ61–195) were purified from E. coli as follows. Starter cultures of BL21(DE3)/RIpL cells harboring plasmids pET22b His6-T7-Mmr1, pET22b His6-T7-Mmr1Δ4E, or pWaldo Mmr1(Δ61–195)-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 OD600 of 0.5 was reached. To induce protein expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 250 µM, and the cultures were grown for 2 h at 30°C for His6-T7-Mmr1 and His6-T7-Mmr1Δ4E and 16 h at 18°C for Mmr1(Δ61–195)-GFP-His8. The cells were then harvested by velocity centrifugation at 5000 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 N2, and stored at −80°C. The cell suspension was quickly

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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 × g for 45 min 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 h at 4°C, and the resin was then pelleted at 3000 × g for 3 min. 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. Each elution (5 µl) was mixed with sample buffer, run on a SDS–PAGE gel, and Coomassie stained. Mmr1, Mmr1ΔCT, or Mmr1(Δ61–195) 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 N2, and stored at −80°C. The concentration of the purified proteins was determined using a bicinechonic acid (BCA) protein assay kit (Pierce).

Liposome flotation assays
The following phospholipids were supplied in chloroform at 10 mg/ml from Avanti Polar Lipids: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho (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-phospho-rac-1-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 (Zinner and Daum, 1995). For OMC ± 0% CL and OMC ± 17% CL, compensatory changes were made in the percent- age 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 h. 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. Four hundred microliters of 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 × g at 4°C for 2 h. 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 MS plate reader ( Molecular Devices) with the excitation and emission monochromators set at 550 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 immunoglobulin G (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 mid-log phase in yeast extract, peptone + 2% (wt/vol) dextrose (YPD) media. For Supplemental Figure S2, B and C, 0.5 mM estradiol and 0.05 mM estradiol were added, as indicated, to drive expression of GalSa::MMR1-yEGFP and GalSa::MMR1ΔC-yEGFP, respectively. Cells (1.0 OD) were harvested, and whole cell extracts were prepared using a NaOH lysis and trichloroacetic acid (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 mid-log phase (−0.8 OD600 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 (ILPB; 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, 1 mM DTT, 1X Protease Inhibitor Cocktail Set 1 (Millipore), and phosphatase inhibitors (60 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium molybdate, 50 µM cantharidin). Prechilled 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. Triton X (0.1%) 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 × g for 30 s. The lysates in the new tubes were centrifuged at 17,000 × g for 30 min at 4°C to remove all large cell debris. Anti-FLAG or anti-GFP μMACS beads (25 µl; 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 ILPB + 0.1% Triton X-100 + PIC. The lysates were added to the equilibrated columns. Columns were washed with 800 µl ILPB + 0.1% Triton X-100 + PIC three times and with 500 µl ILPB, no detergent, no PIC twice. 1X MURB (25 µl) were added to the columns and incubated for 10 min at room temperature. An additional 25 µl 1X MURB was 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 Δmrr1::NATNT2 and PJ69-4Aalpha Δmrr1::NATNT2 were transformed with the indicated Gal4AD and Gal4BD fusions, respectively. PJ69-4A Δmrr1::NATNT2 cells harboring the indicated Gal4AD fusions were then mated with PJ69-4Aalpha Δmrr1::NATNT2 cells harboring the indicated Gal4BD fusions. Dipsoids were selected on SC–LEU–TRP–DEx plates, and protein–protein interactions were assessed by growth on SC–LEU–TRP–ADE–DEx plates at 24°C.

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