

# Mrx6 regulates mitochondrial DNA copy number in *Saccharomyces cerevisiae* by engaging the evolutionarily conserved Lon protease Pim1

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**ABSTRACT** Mitochondrial function depends crucially on the maintenance of multiple mitochondrial DNA (mtDNA) copies. Surprisingly, the cellular mechanisms regulating mtDNA copy number remain poorly understood. Through a systematic high-throughput approach in *Saccharomyces cerevisiae*, we determined mtDNA-to-nuclear DNA ratios in 5148 strains lacking nonessential genes. The screen revealed *MRX6*, a largely uncharacterized gene, whose deletion resulted in a marked increase in mtDNA levels, while maintaining wild type-like mitochondrial structure and cell size. Quantitative superresolution imaging revealed that deletion of *MRX6* alters both the size and the spatial distribution of mtDNA nucleoids. We demonstrate that Mrx6 partially colocalizes with mtDNA within mitochondria and interacts with the conserved Lon protease Pim1 in a complex that also includes Mam33 and the Mrx6-related protein Pet20. Acute depletion of Pim1 phenocopied the high mtDNA levels observed in  $\Delta mrx6$  cells. No further increase in mtDNA copy number was observed upon depletion of Pim1 in  $\Delta mrx6$  cells, revealing an epistatic relationship between Pim1 and Mrx6. Human and bacterial Lon proteases regulate DNA replication by degrading replication initiation factors, suggesting a model in which Pim1 acts similarly with the Mrx6 complex, providing a scaffold linking it to mtDNA.

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Abbreviations used: BFP, blue fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; MCC, Manders colocalization coefficient; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; PCC, Pearson's correlation coefficient; qPCR, quantitative PCR; SD, synthetic dextrose; SI, structured illumination; SSC-H, side scatter height; YPD, yeast extract peptone dextrose; YPG, yeast extract peptone ethanol glycerol; WT, wild type.

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## INTRODUCTION

Mitochondria are endosymbiotic organelles that carry multiple copies of their own genomes, encoding proteins required for oxidative phosphorylation and respiratory metabolism. Mitochondrial DNA (mtDNA) copies are packaged together with several mtDNA-binding proteins to form nucleoids that distribute throughout the mitochondrial network and display a semiregular spacing in the mitochondrial network (Chen and Butow, 2005; Brown et al., 2011; Osman et al., 2015; Jajoo et al., 2016; Lewis et al., 2016). The copy number of mtDNA in each cell is maintained within a narrow range (Chen and Butow, 2005; Clay Montier et al., 2009). Previous studies reported that *Saccharomyces cerevisiae* cells maintain ~40–60 nucleoids, each carrying ~1–2 mtDNA copies (Chen and Butow, 2005), although some reviews cite up to 10 copies per nucleoid (Lipinski et al., 2010). Mammalian cells can contain thousands of nucleoids, depending on tissue type (Williams, 1986; Miller et al., 2003). Recent superresolution-microscopy experiments demonstrated that most nucleoids contain only a single mtDNA copy (Kukat et al., 2011).

Altered levels of mtDNA are linked to a variety of diseases, including neurodegenerative and metabolic diseases and various

types of cancer (Liu *et al.*, 2006; Clay Montier *et al.*, 2009; Ylikallio *et al.*, 2010; Yu, 2011; Kornblum *et al.*, 2013; Mengel-From *et al.*, 2014; Pyle *et al.*, 2016). Furthermore, increasing mtDNA copy number has been suggested to help cells ameliorate the effect of myocardial infarction in mice (Ikeda *et al.*, 2015). Despite their physiological importance, the cellular mechanisms that regulate mtDNA copy number remain poorly understood. Previous genetic screens designed to identify new components that control mtDNA copy number focused on mutants that lead to mtDNA loss and identified numerous components important for mtDNA maintenance (Fukuoh *et al.*, 2014; Zhang and Singh, 2014). However, loss of mtDNA is often caused by secondary effects due to compromised mitochondrial function (Lipinski *et al.*, 2010). Therefore, the question of how mtDNA copy number is regulated to remain within a narrow window has remained largely unanswered.

To elucidate how mtDNA levels are sustained at their physiological set point, we determined mtDNA levels in a yeast deletion library. This screen revealed the gene *MRX6* as a crucial component of mtDNA copy number control.

## RESULTS

### A genetic screen identifies cellular machineries regulating mtDNA copy number

We systematically determined the amount of mtDNA relative to nuclear DNA (nDNA) in 5148 strains of a yeast gene-deletion library generated in S288c cells, each lacking a different nonessential gene (Saccharomyces Genome Deletion Project; Giaever *et al.*, 2002). Mutant colonies grown on rich medium were transferred to nylon membranes, lysed, and hybridized with two different sets of fluorescent probes specific for mtDNA and nDNA, respectively (Figure 1, A and B). We determined the fluorescence intensity of both probes and calculated the mtDNA/nDNA ratio for each mutant. Mutants were classified into three categories: 1) mutants that contained an increased mtDNA/nDNA ratio, 2) mutants that maintained their mtDNA/nDNA ratio similarly to wild-type cells (WT), and 3) mutants that lost the majority or all of mtDNA (Figure 1C; Supplemental Figure 1A; Supplemental Table 1). The following observations indicate that this screen faithfully reports on mtDNA levels: ~80% of mutants falling into the third category were previously described to be respiratory-deficient or defective in maintenance of mtDNA (Supplemental Figure 1B; Supplemental Table 1B). Furthermore, mutants lacking the genes *SML1*, *RRM3*, and *RFX1*, which were previously reported to contain elevated levels of mtDNA (Taylor *et al.*, 2005), were also identified in our analysis as having increased mtDNA/nDNA ratios (Supplemental Table 1A).

Loss or reduction of mtDNA can result in various mitochondrial defects (Lipinski *et al.*, 2010). Therefore, we focused on mutants that displayed higher mtDNA/nDNA ratios, which are more likely to be indicative of a defect in mtDNA–copy number regulation. To validate our hits, we repeated the colony blot hybridization with the initially identified candidates (167 mutants) and selected 91 mutants for which increased mtDNA levels were reproduced for further analysis (Supplemental Figure 1C; Supplemental Table 1C). As yeast colonies on agar plates consist of heterogeneous cell populations that differ in metabolic and respiratory states (Traven *et al.*, 2012), we isolated genomic DNA from the 91 mutants grown in liquid cultures from early to midlogarithmic phase and quantified their mtDNA levels relative to those of WT cells by quantitative PCR (qPCR). A majority of the mutants (73 of 91) showed a >50% increase in the mtDNA/nDNA ratio (Supplemental Table 1D).

In yeast, mtDNA copy number correlates linearly with the length of the mitochondrial network (Osman *et al.*, 2015). Furthermore, mi-

tochondrial network volume correlates linearly with cell volume (Rafelski *et al.*, 2012). For this reason, elevated mtDNA levels could result as a secondary effect of increased cell size, as would be expected, for example, in mutants that affect cell cycle progression (Conrad and Newlon, 1982). To eliminate such mutants from our analyses, we determined the cell size of the 91 candidates by flow cytometry using side-scattered light as a proxy. Indeed, a majority of the mutants showed an increase in cell size (Figure 1D; Supplemental Table 1D). By contrast, nine mutants displayed cell sizes that were within 10% of the value obtained for WT cells, making them likely candidates to be involved in mtDNA–copy number regulation (Figure 1E).

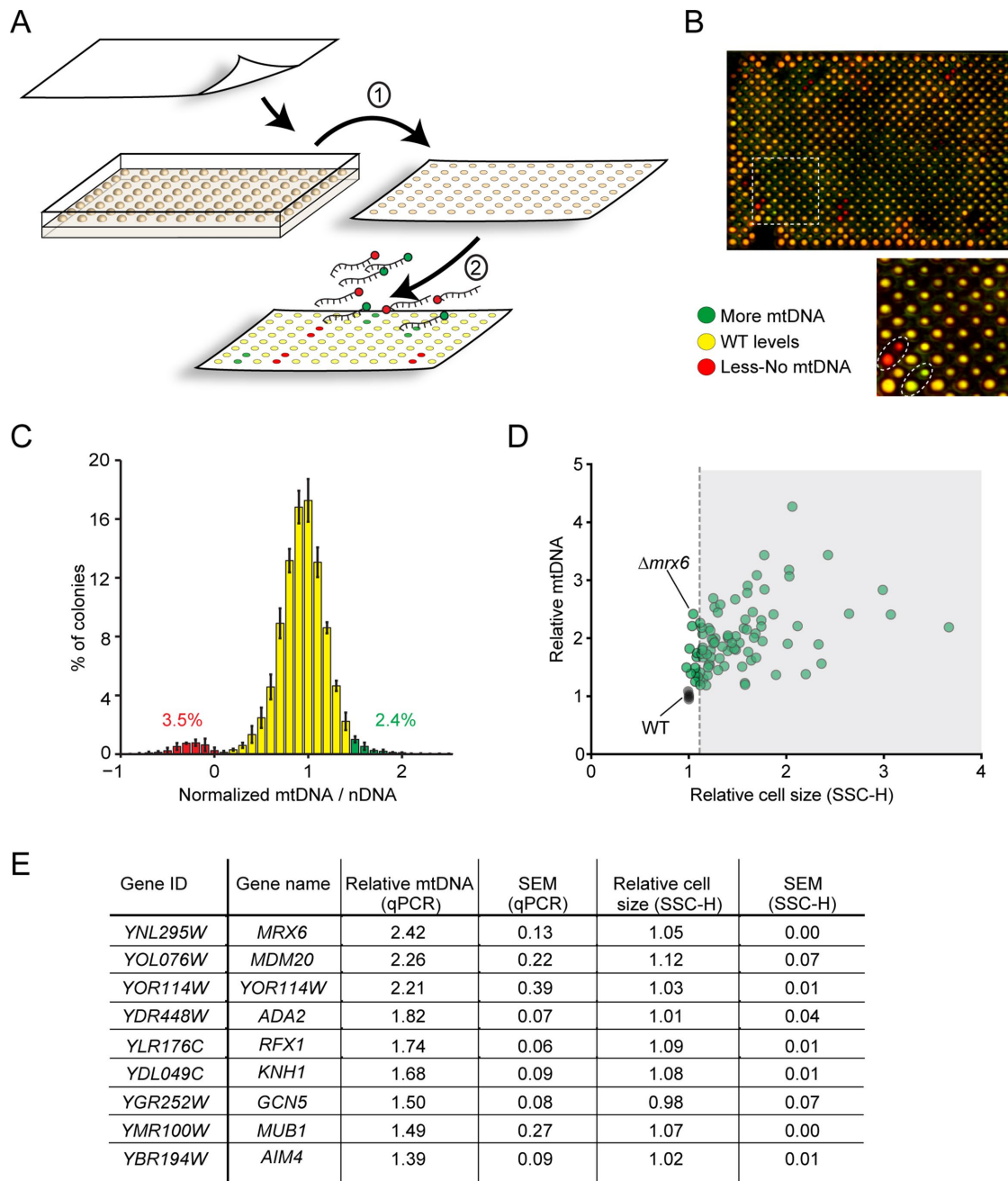
### Deletion of *MRX6*, a largely uncharacterized gene, increases mtDNA copy number

From the genes whose deletion resulted in increased mtDNA levels without altering cell size, we chose to focus on *MRX6* because 1) the mtDNA/nDNA ratio increase in  $\Delta mrx6$  cells was the largest among the mutants that do not affect cell size (Figure 1D), 2) *Mrx6* has a predicted mitochondrial-targeting sequence, and 3) *Mrx6* belongs to an uncharacterized protein family. To verify that increased levels of mtDNA were linked to deletion of *MRX6* and not caused by second-site mutations in the library strain, we engineered a fresh  $\Delta mrx6$  deletion strain, which reproduced the phenotype of strongly elevated mtDNA levels (Figure 2A). While we observed an ~2.5-fold increase in the library strain, we observed only an ~1.5-fold increase in the newly generated strain. We attribute this difference to the fact that the strains were generated in different yeast backgrounds (S288c vs. W303) carrying different amounts of mtDNA (Connelly and Akey, 2012) and/or to a possibility of aggravating second-site mutations in the deletion library strain. The 1.5-fold increase in  $\Delta mrx6$  cells versus WT cells was statistically significant ( $p < 0.01$ ). For the remaining experiments, we used the freshly generated  $\Delta mrx6$  W303 strain.

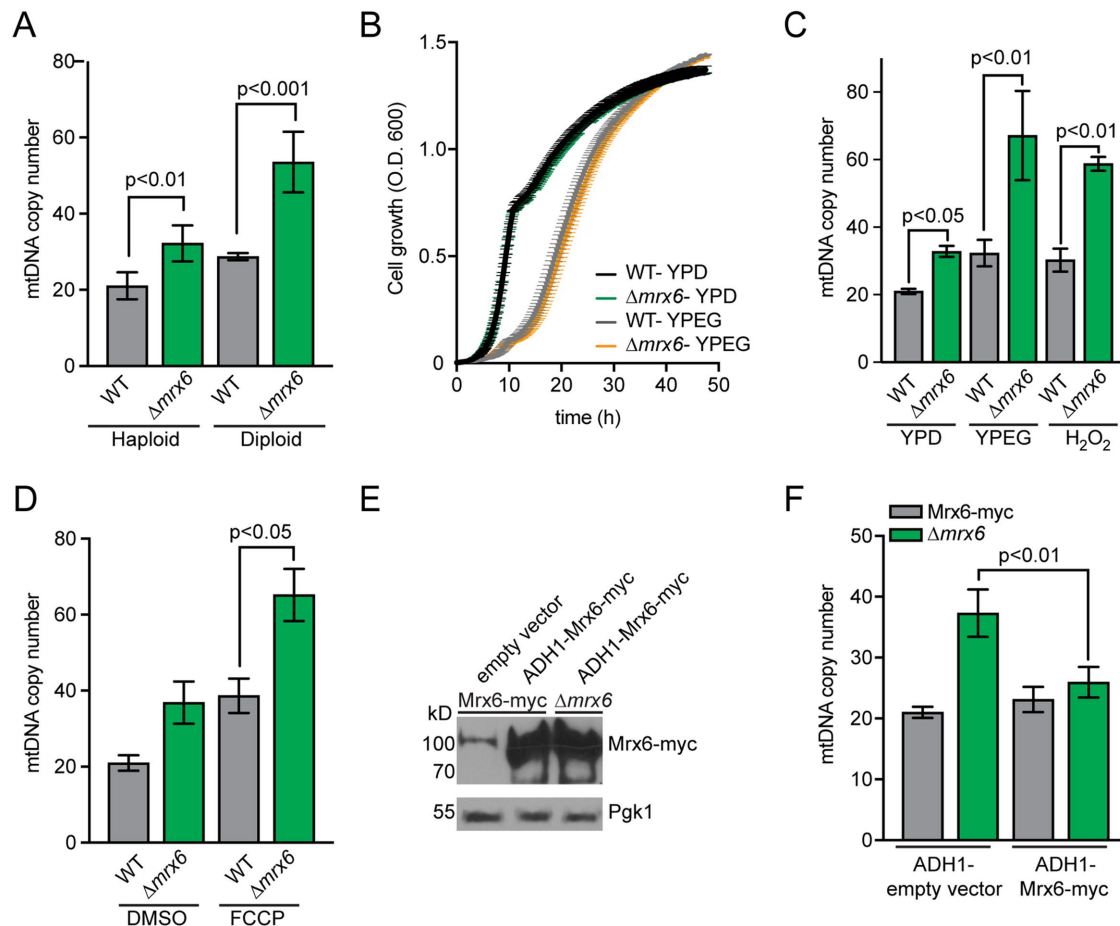
Previous studies reported that mtDNA abundance ranges from 25 to 100 copies per cell depending on the strain and growth conditions (MacAlpine *et al.*, 2000; Chen and Butow, 2005). To obtain an accurate quantification of mtDNA levels in our strains, we determined the absolute mtDNA copy number by qPCR analysis using oligonucleotides specific for nuclearly encoded *ACT1* and mitochondrially encoded *COX1*. To this end, we cloned ~1 kb fragments of *ACT1* and *COX1* into plasmids, which we used as standards to correlate threshold PCR cycle values with copy number. According to these measurements, we conclude that haploid WT cells have 21 ( $\pm 4$ ) copies of mtDNA, whereas  $\Delta mrx6$  cells carry 32 ( $\pm 5$ ) copies (Figure 2A).

We next tested whether deletion of *MRX6* compromises mitochondrial function. To this end, we monitored growth of  $\Delta mrx6$  cells on a nonfermentable carbon source, which necessitates a functional respiratory chain.  $\Delta mrx6$  cells did not exhibit any growth phenotype, in contrast to WT cells (Figure 2B). Next, we tested mtDNA levels in WT and  $\Delta mrx6$  cells grown under different conditions, such as 1) on a nonfermentable carbon source, 2) under oxidizing stress, and 3) in the presence of an electron-transport chain uncoupler (Figure 2, C and D). These analyses revealed that the absence of *MRX6* leads to a robust increase of mtDNA under all conditions tested and that it occurs independent of metabolic or stress adaptation responses.

As deletion of *MRX6* increased mtDNA levels, we next asked whether overexpression of *Mrx6* would decrease them. We overexpressed a C-terminally myc-tagged version of *Mrx6* (*Mrx6*-myc; Supplemental Figure 2) but did not observe altered mtDNA levels



**FIGURE 1:** Forward genetic screen to identify cellular machinery regulating mtDNA copy number. (A) Schematic illustration of the genetic screen. Mutants of the yeast deletion library were grown on agar plates (fermentable rich media, YPD) and transferred to nylon membranes (1), lysed, and hybridized with two sets of fluorescent probes specific for mitochondrial DNA (green) or nuclear DNA (red) (2). (B) Scan of a colony blot is shown as an overlay of two channels. Each mutant has its replicate on the diagonal. Mutants with mtDNA/nDNA ratios similar to WT appear yellow, whereas mutants with increased or decreased mtDNA levels are depicted in green and red, respectively. (C) Histogram showing distribution of relative fold changes in mtDNA/nDNA ratios of the mutants. Error bars indicate standard deviations (SD) of three independent colony blot experiments ( $n = 3$ ). Out of total mutants, 2.4% showed an increase in mtDNA copy number by at least 50% (green) and 3.5% lost the majority of or entirely lack mtDNA (red). mtDNA/nDNA ratios below zero are due to subtraction of colony auto-fluorescence from hybridization signal (Supplemental Figure 1; for the list of mutants see Supplemental Table 1). (D) mtDNA levels of 91 hits identified by colony blot screens were verified by qPCR, shown as an average of two independent experiments. Cell sizes of mutants were determined by flow cytometry using SSC-H. Values are relative to WT (see Supplemental Table 1D). Three mutants showing budding defects were omitted from analysis; WT shown in black. Dashed line marks 10% cutoff. Cells were grown in YPD. (E) List of genes identified in this study; their deletion mutants led to an increase in mtDNA copy number but their cell size remained within 10% of WT. mtDNA levels and cell sizes were determined relative to WT ( $n = 2$ ).



**FIGURE 2:** Deletion of an uncharacterized gene, *MRX6*, increases mtDNA copy number. (A) qPCR analyses of mtDNA copy number in haploid and diploid W303 cells lacking *MRX6*. Cells were grown in YPD. Error bars indicate SD ( $n = 4$ ). (B) Growth curves of WT and  $\Delta mrx6$  cells grown in YPD or YPEG (rich media with ethanol and glycerol;  $n = 2$ ). (C) qPCR analyses of mtDNA levels in WT and  $\Delta mrx6$  cells grown in YPD or YPEG or treated with 0.5 mM  $H_2O_2$  in YPD for 1 h. (YPD and YPD+  $H_2O_2$ ,  $n = 2$ ; YPEG,  $n = 4$ .) (D) qPCR analyses of mtDNA levels in WT and  $\Delta mrx6$  cells grown in YPD and treated with DMSO or FCCP (5  $\mu$ g/ml) for 1 h ( $n = 2$ ). (E) Western blot analyses of Mrx6-myc levels in cells that either express Mrx6-myc or lack Mrx6, transformed with an empty vector or a vector allowing overexpression of Mrx6-myc from the ADH1 promoter. Cells were grown in dropout synthetic media with dextrose, lacking URA (SD-ura). PGK1 was used as a loading control. (F) qPCR analysis of mtDNA copy number in cells overexpressing Mrx6-myc, shown in E ( $n = 4$ ).

(Figure 2, E and F). Mrx6-myc was functional because it maintained mtDNA at WT levels when it was expressed as the only copy of the gene. Deletion of *MRX6* increases mtDNA copy number without compromising mitochondrial function, while conversely overexpression of Mrx6 does not affect mtDNA levels.

### Deletion of *MRX6* increases mtDNA copy number without altering mitochondrial network length or morphology

To test whether deletion of *MRX6* causes abnormalities in mitochondrial volume or morphology, we visualized the mitochondrial network in  $\Delta mrx6$  and WT cells by fluorescence microscopy with a mitochondria-targeted red fluorescent protein (mt-dsRed; Figure 3A). We did not detect any changes in mitochondrial morphology (Figure 3, B and C) or network length (Figure 3D) in  $\Delta mrx6$  cells, demonstrating that elevated mtDNA levels in  $\Delta mrx6$  cells were not a consequence of morphological alterations.

To determine the copy number of mtDNA in single cells, we used our recently developed mt-LacO-LacI system to visualize mtDNA by fluorescence microscopy. The system is based on an

array of LacO repeats integrated into mtDNA, which can be bound by a mitochondria-targeted GFP-tagged LacI protein (Osman *et al.*, 2015). In contrast to our previous work, we applied structured illumination (SI) microscopy on fixed diploid cells to resolve single mtDNA copies that are in close proximity to one another (Figure 3A). We counted ~28 mtDNA copies in WT diploid cells, which is in good agreement with 33 ( $\pm 6$ ) mtDNA copies, determined by qPCR (Figure 2A), indicating that the microscopic analysis resolved single mtDNA copies in the majority of cases for WT cells. Given that cells used in this experiment were not synchronized and were going through different stages of the cell cycle, resulting in differences in cell size and mitochondrial volume, we compared mtDNA copy number normalized to mitochondrial network length. Deletion of *MRX6* increased the number of mtDNA copies normalized to mitochondrial network length 1.3-fold (Figure 3E; WT = 1.16,  $\Delta mrx6$  = 1.54 mtDNA copies/ $\mu$ m network length). A 1.3-fold increase is smaller than what we obtained with qPCR (Figure 2A). We attribute the difference between microscopy and qPCR analysis to the fact that even with SI microscopy, we did not resolve

mtDNA copies that were close to one another (such as replicating mtDNA copies).

Next, we compared the distances between mtDNA copies in the three-dimensional mitochondrial network. Consistent with the increased number of nucleoids within the same length of mitochondrial network, the mean distances between mtDNA copies were 692 and 872 nm for  $\Delta mrx6$  and WT cells, respectively ( $p < 0.001$ ; Figure 3F). However, upon more detailed examination, we found that this difference was largely due to a closer apposition of a subset of mtDNA copies in the smaller distance range, while mtDNA copies further apart maintained their distribution as in WT cells. This bias is quantified in Figure 3G, which shows that mtDNA distances in the range 0.5–1.0  $\mu\text{m}$  were disenriched in the mutant cells as compared with WT cells (Figure 3G, blue box), whereas mtDNA distances below 0.5  $\mu\text{m}$  were enriched (Figure 3G, yellow box). These nonlinear alterations in the placement of mtDNA copies with respect to each other in  $\Delta mrx6$  cells are in line with the observation that the overall mitochondrial network length is not altered in the mutant.

We next stained fixed diploid cells with DAPI (4',6-diamidino-2-phenylindole) and analyzed them by SI microscopy (Figure 4A). In contrast to the LacO/LacI system shown above, DAPI stains mtDNA in its entirety, rather than just demarking a single locus on it. DAPI staining in WT cells revealed distinct punctate structures known as nucleoids (Meeusen and Nunnari, 2003). While  $\Delta mrx6$  cells showed the same number of nucleoids, the average volume of nucleoids was enlarged ~2.2-fold in comparison with WT cells (Figure 4, B and C). We verified this finding using an anti-DNA antibody (Supplemental Figure 3) to ascertain that it was indeed DNA and not other DAPI-stained macromolecules, such as RNA, that gave rise to the increased volume. Notably, nucleoids in  $\Delta mrx6$  cells displayed an oblong shape: their lengths, when traced along the mitochondrial network, were significantly increased (mean lengths of nucleoids were 630 and 430 nm for  $\Delta mrx6$  and WT cells, respectively;  $p < 0.001$ ; Supplemental Figure 3, A and B). Taken together, these data show that deletion of *MRX6* increases mtDNA copy number without affecting mitochondrial network length or shape, but alters the spatial organization and shape of nucleoids.

### **Mrx6 is a member of the PET20 domain-containing protein family**

Interestingly, inspection of the Mrx6 sequence revealed the presence of a PET20 domain of uncharacterized structure and function, which in *S. cerevisiae* is found in two other mitochondrial proteins, Sue1 and Pet20 (Figure 5A). In addition to the PET20 domain, Mrx6 has a unique C-terminal extension that distinguishes it from the rest of the PET20 domain-containing proteins (Supplemental Figure 4). To assess whether other PET20 domain-containing proteins are important for maintaining mtDNA levels, we deleted the genes encoding these proteins in different combinations and quantified the change of mtDNA levels by qPCR. In line with the colony blots from the initial screen, single deletions of *PET20* or *SUE1* did not alter mtDNA levels significantly. Additionally, no further increase in mtDNA levels as compared with those for  $\Delta mrx6$  cells was observed in  $\Delta mrx6 \Delta pet20$ ,  $\Delta mrx6 \Delta sue1$ , and  $\Delta mrx6 \Delta pet20 \Delta sue1$  double and triple mutant strains (Figure 5B). Thus, neither maintenance of normal mtDNA levels nor increase in mtDNA levels in  $\Delta mrx6$  cells requires Pet20 or Sue1.

### **Mrx6 forms foci in mitochondria and colocalizes with mtDNA**

Mrx6 has a predicted mitochondrial-targeting sequence, but to date its localization has not been determined experimentally. We con-

structed a yeast strain in which we genomically tagged Mrx6 in its endogenous locus with the fluorescent protein mNeonGreen (Mrx6-Neon) to determine its localization by fluorescence microscopy. Cells expressing Mrx6-Neon displayed mtDNA levels indistinguishable from those in WT cells, indicating that protein function was preserved in the tagged Mrx6 variant (Supplemental Figure 5A). In agreement with its predicted mitochondrial localization, Mrx6-Neon colocalized with mitochondrial matrix-targeted blue fluorescent protein (mtTagBFP; Figure 6A). Interestingly and in contrast to mt-TagBFP, Mrx6-Neon formed discrete punctate structures that were nonuniformly distributed along the mitochondrial network.

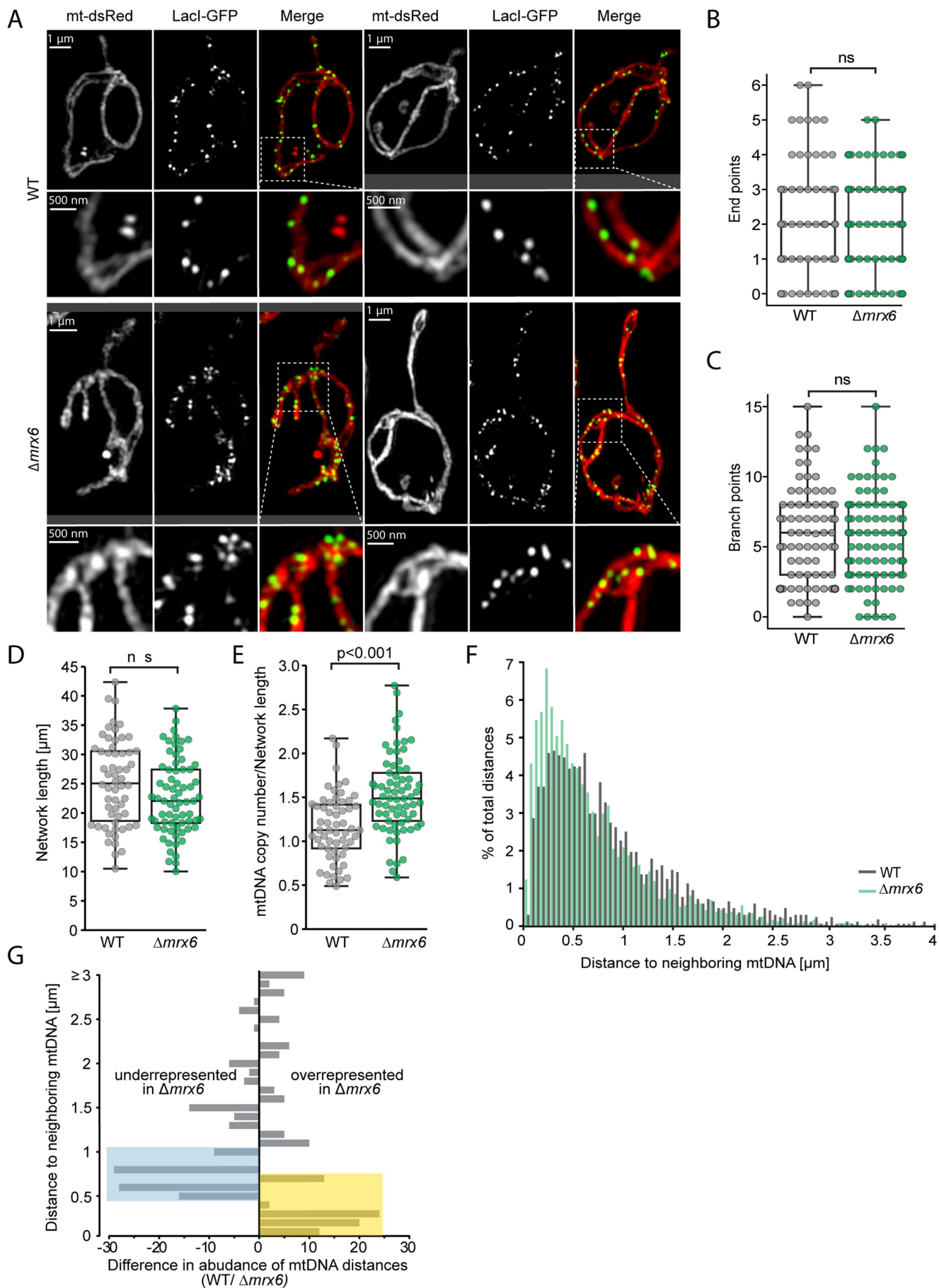
The punctate localization of Mrx6-Neon was reminiscent of the distribution of mtDNA in the nucleoids in the mitochondrial network. Thus, we next tested whether Mrx6-Neon colocalizes with mtDNA. We stained Mrx6-Neon-expressing cells with DAPI and determined Mrx6-Neon and mtDNA localization. These analyses revealed that a fraction of Mrx6-Neon puncta colocalized with the DAPI signal (Figure 6B; arrow), whereas others did not (Figure 6B; asterisk). We quantitatively assessed the proportion of the Mrx6-Neon signal that colocalized with DAPI and vice versa by determining the Manders colocalization coefficient (MCC) between intensity profiles of the two fluorescent signals along the mitochondrial network (Figure 6C and Supplemental Figure 5B). The MCC values showed a broad distribution, yet the majority of cells showed ~60% overlap between the two wavelengths, confirming our initial observation of partial colocalization between Mrx6 and mtDNA (Figure 6D). Pearson's correlation coefficient (PCC) analysis further supported colocalization (Figure 6E). To validate these conclusions, we evaluated the significance of the measured MCC and PCC values by comparing our results with a control data set. This data set consisted of the same intensity profiles, in which one of the two color channels was randomized (Supplemental Figure 5C). The control MCC and PCC values were consistently lower and showed a distribution significantly different from that of the measured data (Figure 6, D and E, and Supplemental Figure 5D;  $p < 0.001$ ). Additionally, comparing the measured MCC and PCC values to a simulated data set in which one of the two channels was shifted against the respective other channel, rather than randomized, further validated these results.

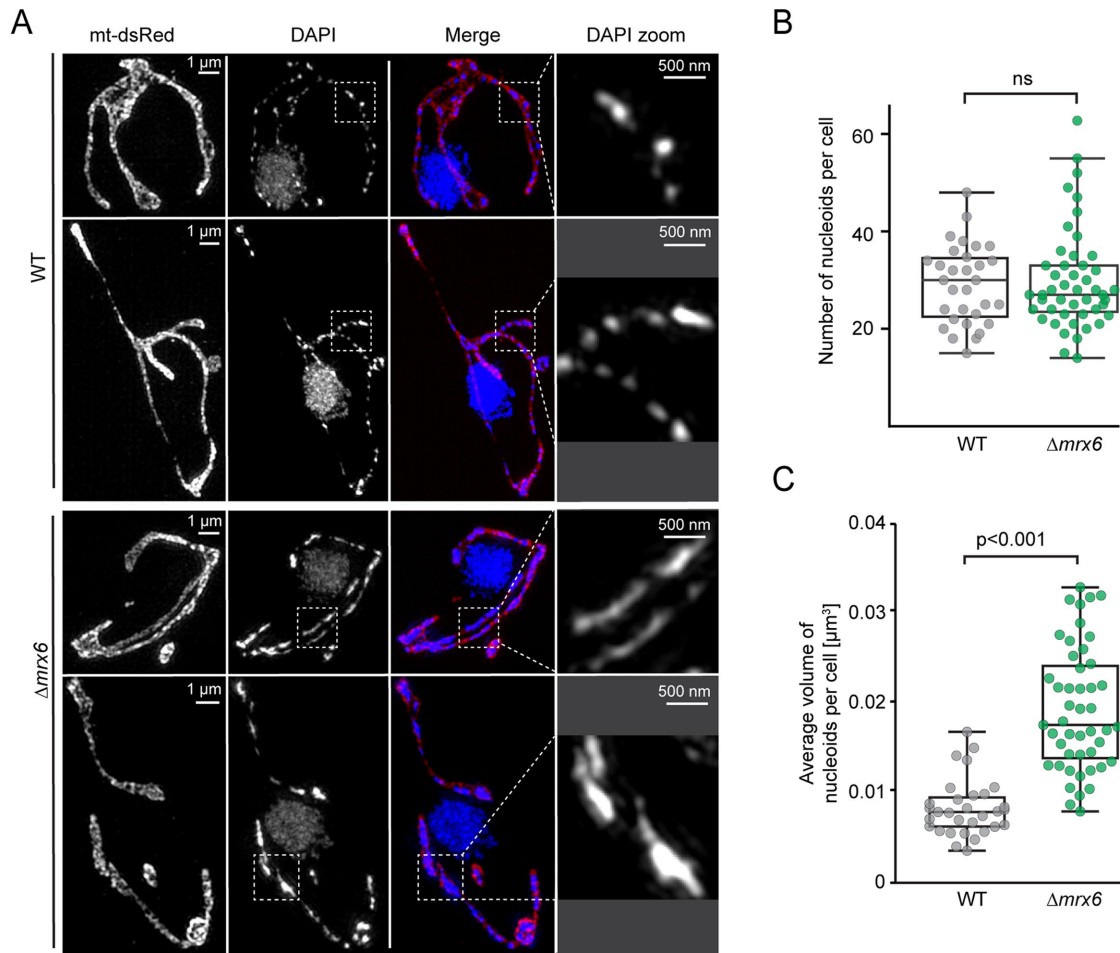
Given that only ~60% of the Mrx6-Neon puncta colocalized with nucleoids, we asked whether Mrx6 might still forms punctate structures in the absence of mtDNA. Mrx6-Neon was still observed in puncta in mitochondria of mtDNA-lacking cells ( $\rho^0$ ; Figure 6F). Taken together, these data show that Mrx6 localizes to mitochondria and forms puncta that distribute throughout the mitochondrial matrix and partially colocalize with mtDNA. However, the presence of mtDNA is not necessary for the formation of Mrx6 puncta.

### **Mrx6 forms a complex with Pet20, Mam33, and Pim1**

Next, we aimed to identify interaction partners of Mrx6 to begin getting a molecular understanding of how Mrx6 affects mtDNA levels. To this end, we immunoprecipitated functional C-terminally Flag-tagged Mrx6 (Mrx6-Flag, Supplemental Figure 6) and identified interacting proteins by mass spectrometry (MS). The proteins Pim1, Mam33, and, to our surprise, Pet20 copurified with Mrx6-Flag but were absent in the eluate fraction of control immunoprecipitations from cells that only expressed untagged Mrx6 (Figure 7, A and B).

Pim1 is a highly conserved ATP-dependent mitochondrial Lon protease (Venkatesh et al., 2012), and Mam33 is a specific translational activator of Cox1 mRNA (Roloff and Henry, 2015). We next asked whether, reciprocally, we could copurify these components by pulling down C-terminally Flag-tagged Pet20 (Pet20-Flag).





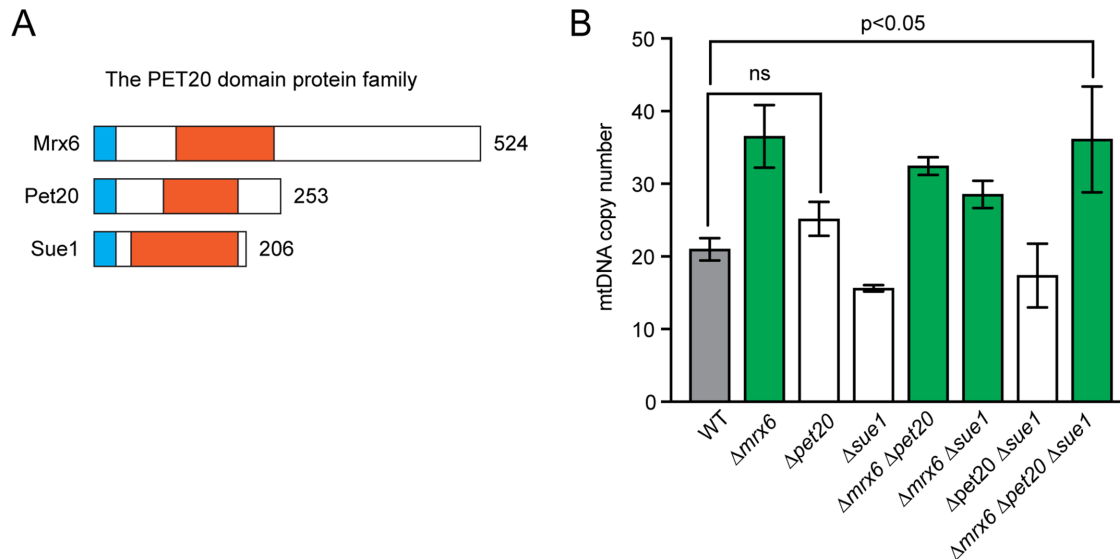
**FIGURE 4:**  $\Delta mrx6$  cells display elongated nucleoids. (A) Z-projections of SI microscopic images of paraformaldehyde and methanol-fixed diploid cells that were stained with DAPI. Mitochondria were visualized by mt-dsRED. Cells were grown in SD-ura-trp. (B) Number and (C) average volume of nucleoids stained with DAPI in WT and  $\Delta mrx6$  cells. Analysis was performed on three-dimensional images (31 cells for WT, 47 cells for  $\Delta mrx6$ ).

Strikingly, Mrx6, Pim1, and Mam33 copurified with Pet20-Flag, thus revealing an interaction network between these four proteins (Figure 7C). We further examined the interaction hierarchy of these proteins by pulling down Mrx6-Flag from extracts of  $\Delta pet20$  cells. The results showed that Pet20 was dispensable for the interaction between Mrx6-Pim1 and Mrx6-Mam33 (Figure 7B). In contrast, upon Pet20-Flag pull down from  $\Delta mrx6$  lysates, Pim1-Pet20 and Mam33-Pet20 interactions were drastically reduced and not detected, respec-

tively, suggesting that Mrx6 bridges Pim1, Mam33, and Pet20 (Figure 7C).

Because two proteins of the PET20 domain protein family, Mrx6 and Pet20, are found in a protein interaction network together with Pim1 and Mam33, we asked whether the third member of the PET20-domain protein family, Sue1, would show a similar protein interaction profile. To this end, we immunoprecipitated C-terminally Flag-tagged Sue1 (Sue1-Flag). In agreement with the Mrx6-Flag

**FIGURE 3:** Deletion of *MRX6* increases mtDNA copy number without altering mitochondrial network length and morphology. (A) Z-projections of SI microscopic images of paraformaldehyde-fixed diploid WT and  $\Delta mrx6$  cells. Mitochondria were visualized by mitochondria-targeted dsRed protein (mt-dsRed). LacI-GFP marks mtDNA. Cells were grown in SD-ura-trp. (B–E) Number of end points (B), branch points (C), length of mitochondrial network (D), and mtDNA copy number normalized to mitochondrial network length (E) in WT and  $\Delta mrx6$  cells. Analysis was performed on three-dimensional images (58 cells for WT, 69 cells for  $\Delta mrx6$ ). (F) Histogram showing distribution of distances between neighboring mtDNA copies in WT and  $\Delta mrx6$  cells in three-dimensional images. Means of distance between mtDNA copies are 692 and 872 nm for  $\Delta mrx6$  and WT cells, respectively ( $p < 0.001$ ). (G) Histogram depicting the differences in the abundance of distances between neighboring mtDNA copies (binning range 0.1  $\mu\text{m}$ ) between the observed WT distribution ( $n = 1677$ ) and a remodeled  $\Delta mrx6$  distribution. In the remodeled  $\Delta mrx6$  distribution all determined distances in mutant cells ( $n = 1677$ ) were multiplied by a factor of  $\sim 1.28$  assuming that distances would be only shifted linearly to closer distances in  $\Delta mrx6$  cells than in WT. Numbers of neighboring mtDNA copies in each binned distance range in the remodeled  $\Delta mrx6$  distribution were replaced by the respective amounts determined for the WT distribution. The distance range from 0 to 0.7  $\mu\text{m}$  that is overrepresented in the remodeled  $\Delta mrx6$  distribution is highlighted in yellow; the one underrepresented between 0.5 and 1  $\mu\text{m}$  is highlighted in blue. The remodeled  $\Delta mrx6$  distribution is significantly different from the WT distribution ( $\chi^2 = 52.67$ ,  $df = 29$ ,  $p < 0.005$ ).



**FIGURE 5:** Mrx6 is a member of the PET20 domain–containing protein family. (A) Domain architecture of Mrx6 and other PET20 domain–containing proteins. Blue indicates mitochondrial targeting sequences. Orange represents PET20 domain. (B) Analysis of mtDNA copy number of single-, double-, and triple-deletion mutants of *MRX6*, *PET20*, and *SUE1*, measured by qPCR. Error bars are SD ( $n = 2$ ). Cells were grown in YPD.

and Pet20-Flag pull downs, which did not identify Sue1, neither Mrx6 nor Pet20 coimmunoprecipitated with Sue1-Flag. However, this experiment revealed that Pim1 also interacts with Sue1 (Figure 7D). Thus, Pim1 is a common interaction partner of all three PET20 domain–containing proteins.

Taken together, these results show that Mrx6, Pet20, Pim1, and Mam33 are part of a physical interaction network in which Mrx6 is crucial for the complex’s architecture, whereas Pet20 is dispensable. In addition, our results support the conclusion that Sue1 forms a separate complex with Pim1, which does not include Mrx6 or Pet20.

### Mrx6 partially colocalizes with Pet20 and Pim1

As Mrx6 and Pet20 are part of an interaction network, we next examined the spatial association between them in single cells. To this end, we engineered a yeast strain expressing Mrx6-Neon and C-terminally mRuby-tagged Pet20 (Pet20-Ruby) and performed live-cell microscopy.

Pet20-Ruby showed discrete punctate structures along the mitochondrial network similar to those for Mrx6 (Figure 8A). Surprisingly, we observed only partial colocalization between Mrx6 and Pet20, in which only some of the Mrx6-Neon puncta colocalized with Pet20-Ruby (Figure 8A, arrow), and vice versa. MCC values showed ~50% overlap between Mrx6-Neon and Pet20-Ruby signals (Figure 8B and Supplemental Figure 7A;  $p < 0.001$ ), and PCC analysis confirmed their colocalization (Figure 8C;  $p < 0.001$ ).

We next tested whether Mrx6 would display a similar partial colocalization with Pim1, a notion suggested by our finding that Pim1 forms a separate complex with Sue1 that lacks Mrx6 or Pet20 (Figure 7D). Hence, we analyzed the association between Mrx6 and Pim1 using a yeast strain expressing Mrx6-Neon and C-terminally mRuby-tagged Pim1 (Pim1-Ruby), which preserves protein function (Supplemental Figure 7B). We observed partial colocalization between Mrx6 and Pim1 (Figure 8D). MCC values showed ~50% overlap between Mrx6-Neon and Pim1-Ruby signals (Figure 8E and Supplemental Figure 7C;  $p < 0.001$ ), and PCC analysis further supported their colocalization (Figure 8F;  $p < 0.001$ ). Taken together, these data indicate that Mrx6 partially colocalizes with Pet20 and Pim1,

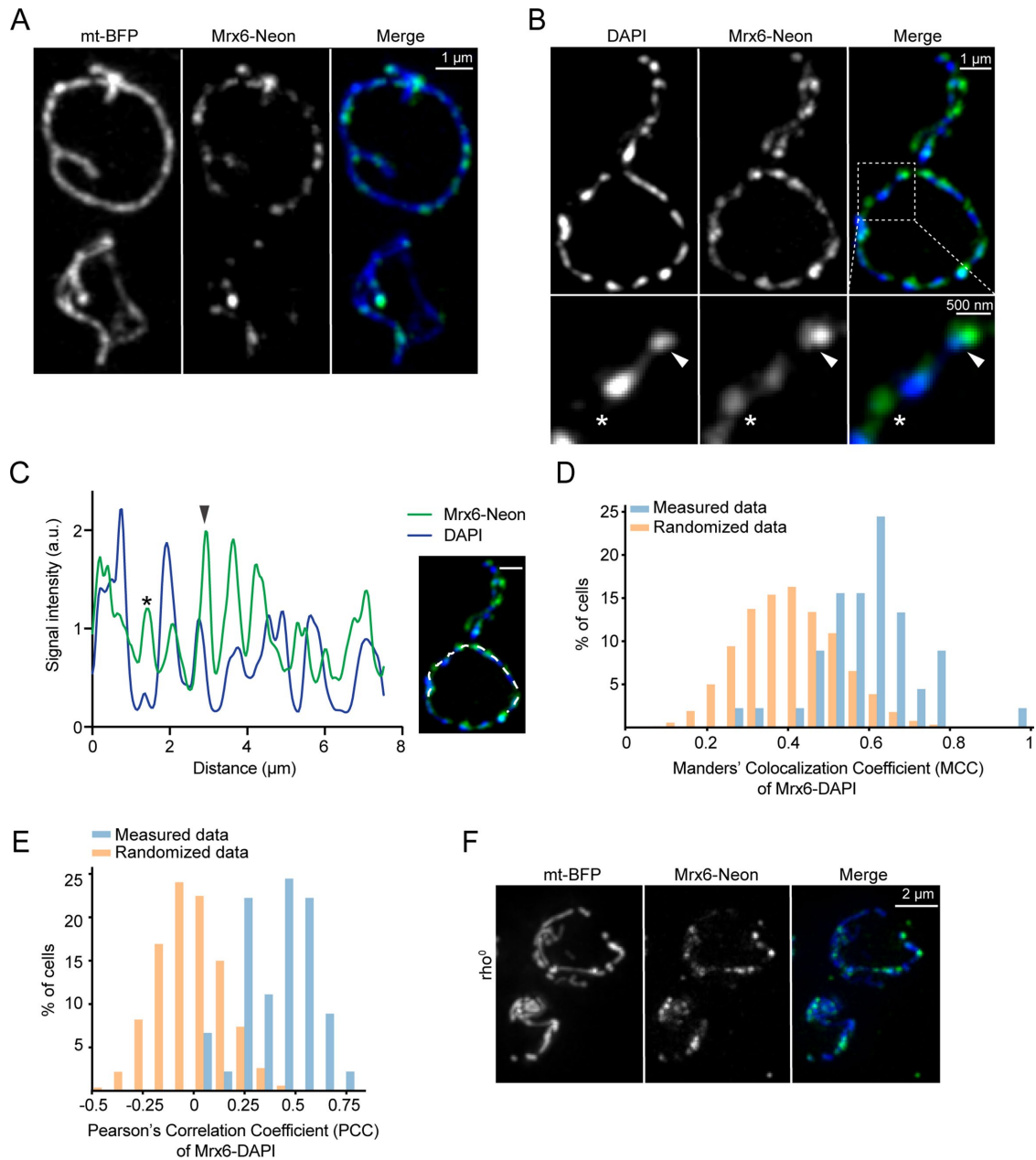
suggesting that they form subcomplexes along the mitochondrial network.

### The Mrx6 complex colocalizes with mtDNA

We next tested whether colocalization of Mrx6 with Pet20 and Pim1 may occur preferentially in regions close to mtDNA. To this end, we performed triple labeling experiments in which we stained mtDNA with DAPI in cells expressing Mrx6-Neon and Pet20-Ruby. This experiment revealed instances of colocalization between Mrx6-Neon, Pet20-Ruby, and mtDNA (Figure 9A). The complexity of images displaying three colors in the confined space of mitochondria necessitated careful quantification of the degree of colocalization. To this end, we binned regions of the Mrx6-Neon and Pet20-Ruby intensity profiles where 1) both proteins colocalized, 2) only Mrx6-Neon but not Pet20-Ruby localized, and 3) only Pet20-Ruby but not Mrx6-Neon localized. We then asked whether these regions would colocalize differentially with mtDNA. Determination of the MCC values revealed that in Bin 1 (Mrx6-Pet20), on the average, 77% of the regions colocalized with DAPI (Figure 9B and Supplemental Figure 8A). In contrast, in Bin 2 (Mrx6 alone) or Bin 3 (Pet20 alone) only 56% or 42% of the regions, respectively, colocalized with DAPI, which was significantly different from the result for Bin 1 ( $p < 0.001$ ; Figure 9B and Supplemental Figure 8A). As a control, we generated a data set by randomizing the DAPI intensity profile against the Mrx6 and Pet20 profiles and recalculated the MCC values. Strikingly, the proportion of the Mrx6-Pet20 signal colocalizing with the DAPI signal was reduced to 44% (Figure 9C and Supplemental Figure 8B), matching the colocalization with mtDNA observed for Mrx6 alone (45%; Bin 1 – Bin 2,  $p = 0.78$ ; Figure 9C) or Pet20 alone (44%; Bin 1 – Bin 3,  $p = 0.79$ ; Supplemental Figure 8B). These results suggest that Mrx6-Pet20 colocalization occurs preferentially in regions close to mtDNA rather than areas that are devoid of mtDNA.

In an analogous set of experiments, we examined the colocalization between Mrx6-Neon, Pim1-Ruby, and mtDNA (Figure 9D). The MCC values revealed that on the average 65% of the areas in which Mrx6-Neon and Pim1-Ruby were found together colocalized with

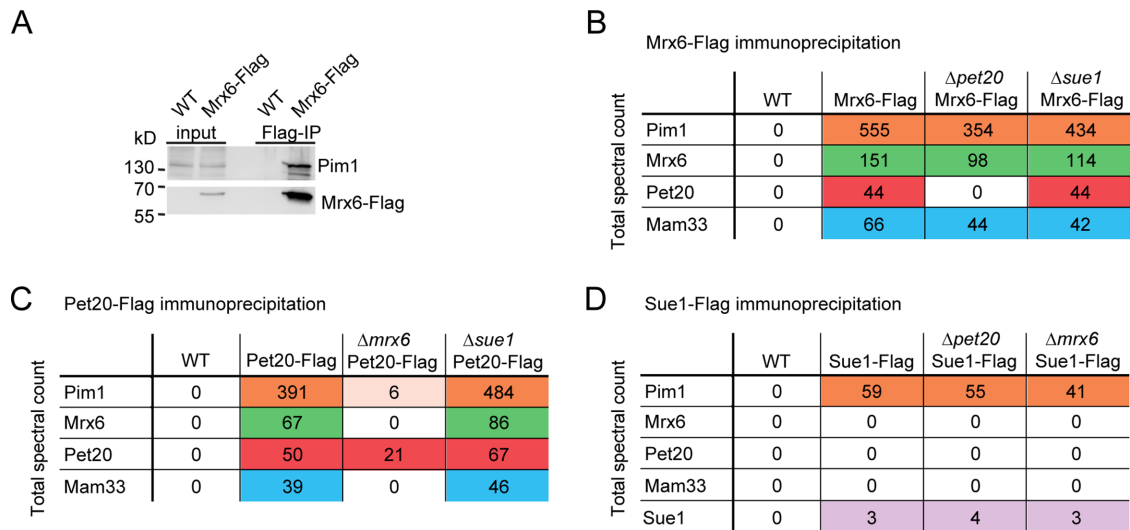




**FIGURE 6:** Mrx6 forms foci in mitochondria and colocalizes with mtDNA. (A) Z-projection of microscopic images of a live cell expressing Mrx6-Neon. Mitochondria were visualized by mt-BFP. Mrx6-Neon (green), mt-BFP (blue) in the merged image. Cells were grown in SD-ura-trp. (B) Z-projections of microscopic images of a live cell expressing Mrx6-Neon (green). mtDNA was stained with DAPI (blue). Arrowhead shows colocalization of Mrx6-Neon and DAPI; asterisk marks noncolocalization. Cells were grown in SD-trp. (C) Linescan analysis of mitochondrial network for the cell shown in B. (D) Distribution of MCC calculated for colocalization of Mrx6-Neon with DAPI in 47 cells (mean = 0.60) or in the same linescans where the DAPI signal was randomized (mean = 0.42,  $p < 0.001$ ). (E) Distribution of PCC determined for Mrx6-Neon and DAPI line cans in measured data ( $n = 47$  cells, mean = 0.40) and in randomized data (mean = 0,  $p < 0.001$ ). (F) Z-projection of microscopic images of a live cell that lacks mtDNA but expresses Mrx6-Neon (green). Mitochondria were visualized by mt-BFP (blue). Cells were grown in SD-ura.

the DAPI signal (Figure 9E and Supplemental Figure 8C); whereas only 46% of the Mrx6 signal ( $p < 0.001$ ; Figure 9E) and 36% of the Pim1 signal colocalized with the DAPI signal when they were alone ( $p < 0.001$ ; Supplemental Figure 8C). Moreover, the percentage of the Mrx6-Pim1 signal colocalizing with the randomized DAPI signal decreased to 38% (Figure 9F and Supplemental Figure 8D), closely matching the values for Mrx6 alone (37%;  $p = 0.49$ ; Figure 9F) and

Pim1 alone (38%;  $p = 0.29$ ; Supplemental Figure 8D). These data indicate that similarly to that of Mrx6-Pet20, Mrx6-Pim1 colocalization occurs in areas close to mtDNA. Taken together, our data suggest that Mrx6 associated with Pet20 or Pim1 preferentially colocalizes with mtDNA, whereas the individual components—or yet-to-be-defined partially assembled subcomplexes—are mostly found in the areas devoid of mtDNA.



**FIGURE 7:** Mrx6 binds to Pet20, Pim1, and Mam33. (A) Western blot analyses of an anti-Flag immunoprecipitation experiment from cells expressing Mrx6-Flag or Mrx6 (WT). Membranes were probed with antibodies against Pim1 (top) and the Flag epitope (bottom). Cells were grown in YPD. (B) Interaction partners of Mrx6-Flag in cells expressing Mrx6-Flag but lacking Pet20 or Sue1, identified by anti-Flag immunoprecipitation and mass spectrometry analysis. The numbers represent total spectral count. Cells were grown in YPD. (C) Interaction partners of Pet20-Flag in cells expressing Pet20-Flag but lacking Mrx6 or Sue1. Same as above. (D) Interaction partners of Sue1-Flag in cells expressing Sue1-Flag but lacking Mrx6 or Pet20. Same as above.

### Depletion of Pim1 increases mtDNA levels

Our biochemical and microscopic analyses suggest that Mrx6 may determine mtDNA levels via its interaction with Pim1. To test this model, we examined mtDNA levels in cells overexpressing or lacking Pim1 by qPCR. Overexpression of Pim1 in WT and  $\Delta mrx6$  cells reduced mtDNA copy number (Supplemental Figure 10A), supporting a putative role of Mrx6 as a regulatory component conferring substrate specificity to Pim1. However, when *PIM1* was deleted, mtDNA copy numbers varied widely in different  $\Delta pim1$  and  $\Delta pim1 \Delta mrx6$  clones, preventing an unequivocal interpretation (Supplemental Figure 10B). These inconsistent results are likely explained by the dysregulated accumulation of the many Pim1 substrates (Major *et al.*, 2006; Bayot *et al.*, 2010), leading pleiotropically to mitochondrial dysfunction.

To mitigate such pleiotropic effects, we integrated a construct facilitating estradiol-dependent expression of Pim1 into WT yeast strains and subsequently generated endogenous deletions of either *PIM1* ( $\Delta pim1$  *Pestr-PIM1*) or *MRX6* and *PIM1* ( $\Delta mrx6 \Delta pim1$  *Pestr-PIM1*). Pim1 expression was maintained throughout strain generation by growing cells in the presence of estradiol. Estradiol concentrations of 25 nM were sufficient to restore respiratory growth in  $\Delta pim1$  background under the experimental conditions at 30°C (Figure 10C) and also resulted in low expression levels that allowed fast Pim1 depletion upon estradiol removal (Figure 10B).

We acutely depleted Pim1 by shifting cells to a medium lacking estradiol and determined mtDNA levels over the course of 20 h (Figure 10, A and B). In line with the low expression level of Pim1 at the start of the experiment, mtDNA levels were increased ~1.3-fold in  $\Delta pim1$  *Pestr-PIM1* cells from those in WT. When Pim1 was depleted, 12 h after removal of estradiol, mtDNA levels were increased 1.8-fold in  $\Delta pim1$  *Pestr-PIM1* cells (Figure 10A). Similarly increased levels were detected in the  $\Delta mrx6 \Delta pim1$  *Pestr-PIM1* double mutant, even at the start of the experiment, when Pim1 was still present. Strikingly, over the time course, mtDNA levels did not increase further in cells lacking both proteins. This strongly

supports a model in which Mrx6 acts via Pim1 to maintain WT mtDNA copy numbers.

### DISCUSSION

We identified new cellular components that modulate mtDNA levels in yeast using a forward genetic screen. We examined mtDNA levels in the 5148 mutants of a yeast deletion library and found that ~2% of these mutants had elevated levels of mtDNA relative to WT. Remarkably, the vast majority of these mutants (~85%) displayed less than a 2.5-fold increase in mtDNA levels, suggesting that a single gene deletion is not sufficient to alter mtDNA levels more drastically. This finding suggests that mtDNA copy number is under stringent regulation, which may be explained by a multilayered system that involves a combination of various components, including factors regulating mtDNA replication and/or stability. One such layer that affects mtDNA levels is cell size, as the majority of our hits with elevated mtDNA copy number displayed increased cell size. This finding supports the notion that mtDNA copy number scales with mitochondrial network length, which in turn scales with cell volume (Rafelski *et al.*, 2012; Osman *et al.*, 2015). Therefore, in contrast to the nuclear genome, mtDNA copy number appears not to be determined on a “per cell” basis, but rather on a “per cell volume” basis. It remains an exciting task for future studies to unravel the molecular basis underlying the coordination between mitochondrial volume, mtDNA copy number, and cell size.

For the nine mutants that displayed elevated mtDNA levels and unaltered cell size, we examined the role of Mrx6 in maintenance of mtDNA levels. Mrx6 has been previously identified in a complex with the mitochondrial ribosome and named “Mitochondria organization of gene expression 6” (Kehrein *et al.*, 2015). However, to date the function of Mrx6 has remained obscure. Based on the following observations, Mrx6 appears directly linked to mtDNA copy-number regulation: 1)  $\Delta mrx6$  cells do not display a growth defect on a nonfermentable carbon source, ruling out the conclusion that mtDNA levels are elevated due to a compensatory feedback

loop that responds to a defective respiratory chain; 2)  $\Delta mrx6$  cells respond to a change in carbon source and display elevated mtDNA levels relative to WT on both fermentable and nonfermentable carbon sources, excluding the possibility that  $\Delta mrx6$  cells are defective in glucose repression (Ulery *et al.*, 1994); 3)  $\Delta mrx6$  cells do not exhibit any changes in mitochondrial network length or morphology, which excludes the possibility that increased mtDNA levels are caused by compromised mitochondrial structure. Thus, elevated mtDNA levels in  $\Delta mrx6$  cells are not simply secondary effects caused by mitochondrial or cellular dysfunction.

Interestingly, deletion of *MRX6* resulted in elongated nucleoids. One plausible explanation for this phenotype is that newly replicated copies of mtDNA in  $\Delta mrx6$  cells remain at least partially associated with parental mtDNA, resulting in bigger and misshapen nucleoids. The oblong shape of nucleoids is reminiscent of the proposed nucleoid division defects in *HSP60* mutants (Kaufman *et al.*, 2003). In line with this notion, we observed clustering of mtDNA copies when mtDNA was visualized with the mt-LacO-LacI system by structured illumination microscopy, which allowed detection and analysis of the spatial distribution of individual mtDNA copies. Alternatively, deletion of *MRX6* could change the packaging of nucleoids, resulting in less compact and elongated nucleoids, which could alter mtDNA levels, perhaps by providing more access to the origins of replication.

*Mrx6* contains a PET20 domain of uncharacterized structure and function that is conserved in fungi, including distant species such as *Schizosaccharomyces pombe*. Two other proteins in *S. cerevisiae*, *Pet20* and *Sue1*, also belong to the PET20 protein family and localize to mitochondria (Wei and Sherman, 2004; Polevoda *et al.*, 2006). We see no alterations in mtDNA levels upon deletion of *PET20* or *SUE1*, alone or in combination, suggesting that both genes either are required in other molecular contexts or only affect mtDNA copy number regulation to minor extents.

Our protein-interaction studies revealed a complex network composed of the PET20 domain-containing proteins and the proteins *Mam33* and *Pim1*. *Mam33* was identified as an *Mrx6* interaction partner and is a specific translational activator of *Cox1* mRNA (Roloff and Henry, 2015). The physiological role of the interaction between *Mrx6* and *Mam33* is currently unknown. However, the interaction points to regulatory coordination between translation and mtDNA copy number, which remains to be clarified in future studies. Interestingly, such coordination has recently been proposed for *SLIMP*, a specialized mitochondrial aminoacyl t-RNA synthetase paralogue, which affects mtDNA levels and interacts with the *Pim1* Lon homologue in arthropods (Picchioni *et al.*, 2019).

Remarkably, *Mrx6*, *Pet20*, and *Sue1* are found in protein complexes that contain the conserved mitochondrial Lon protease *Pim1*, which reveals a strong link between the PET20 domain-containing proteins and the mitochondrial-protein quality-control system. The finding that copurification of *Pim1* with *Pet20* depends on *Mrx6* and that *Mrx6* copurifies with *Pet20* suggests that *Pim1*, *Mrx6*, and *Pet20* may be part of the same complex. In contrast, *Sue1* forms an alternate complex with *Pim1*.

We consider it an attractive possibility that PET20 domain-containing proteins serve as substrate specificity factors for *Pim1*. In agreement, *Sue1* is required for degradation of labile forms of cytochrome *c* (Wei and Sherman, 2004), suggesting that *Sue1*-*Pim1*-dependent proteolysis could play a role in degradation of altered forms of cytochrome *c* (albeit it remains a paradox how misfolded cytochrome *c* would venture into the mitochondrial matrix space to meet its fate or, vice versa, how *Sue1*-*Pim1* might gain access to the intermembrane space to hunt for misfolded cytochrome *c*). By anal-

ogy, *Mrx6* may be important for *Pim1*-dependent degradation of proteins regulating mtDNA copy number. In support of this idea, we found that acute depletion of *Pim1* results in elevated mtDNA levels matching those observed in  $\Delta mrx6$  cells. However, depletion of *Pim1* in  $\Delta mrx6$  cells did not lead to a further increase in mtDNA copy number, thus revealing an epistatic relationship between *Mrx6* and *Pim1*. These observations strongly support our hypothesis that *Mrx6* acts through *Pim1* to regulate mtDNA levels.

Although we consider it an attractive hypothesis, we currently have no direct evidence that mtDNA copy number is affected through the proteolytic activity of *Pim1*. However, in support of this notion, *Pim1* has been shown to degrade multiple proteins involved in mtDNA metabolism (Bayot *et al.*, 2010). For example, *Abf2*, a mtDNA packaging protein required for mtDNA stability, has been reported to be a substrate of *Pim1* and changes in *Abf2* protein levels alter mtDNA copy number (Zelenaya-Troitskaya *et al.*, 1998; Bayot *et al.*, 2010). Moreover, the orthologue of *Abf2* in higher eukaryotes, *TFAM*, modulates mtDNA levels (Ekstrand *et al.*, 2004; Kanki *et al.*, 2004), and changes in Lon protease expression alter mtDNA copy number through degradation of *TFAM* (Matsushima *et al.*, 2010; Lu *et al.*, 2013). In our hands, however, in  $\Delta mrx6$  cells' overall *Abf2* protein levels did not change relative to WT cells, suggesting that the increased mtDNA phenotype in  $\Delta mrx6$  cells relies on subtle local changes if *Abf2* is an *Mrx6*-dependent *Pim1* substrate (Supplemental Figure 10). Similarly, the human mitochondrial protease Lon degrades the DNA helicase *Twinkle* (Kunova *et al.*, 2017). Therefore, mtDNA copy number regulation by *Mrx6*-*Pim1*-dependent proteolysis might not be limited to a single substrate.

*Pim1* colocalizes with nucleoids (Kunova *et al.*, 2017), and its human homologue binds to mtDNA, preferentially in the control region where mtDNA transcription and replication are initiated (Lu *et al.*, 2007). While our localization studies support an *Mrx6*-dependent link between *Pim1* and mtDNA, our attempts to footprint the *Pim1*-*Mrx6* complex on mtDNA by chromatin immunoprecipitation have been unsuccessful. Interestingly, we found *Mrx6* complexes containing *Pim1* and/or *Pet20* in the close vicinity of mtDNA, whereas the fraction of *Mrx6* not colocalizing *Pim1* and/or *Pet20* was found predominately in DNA-free areas.

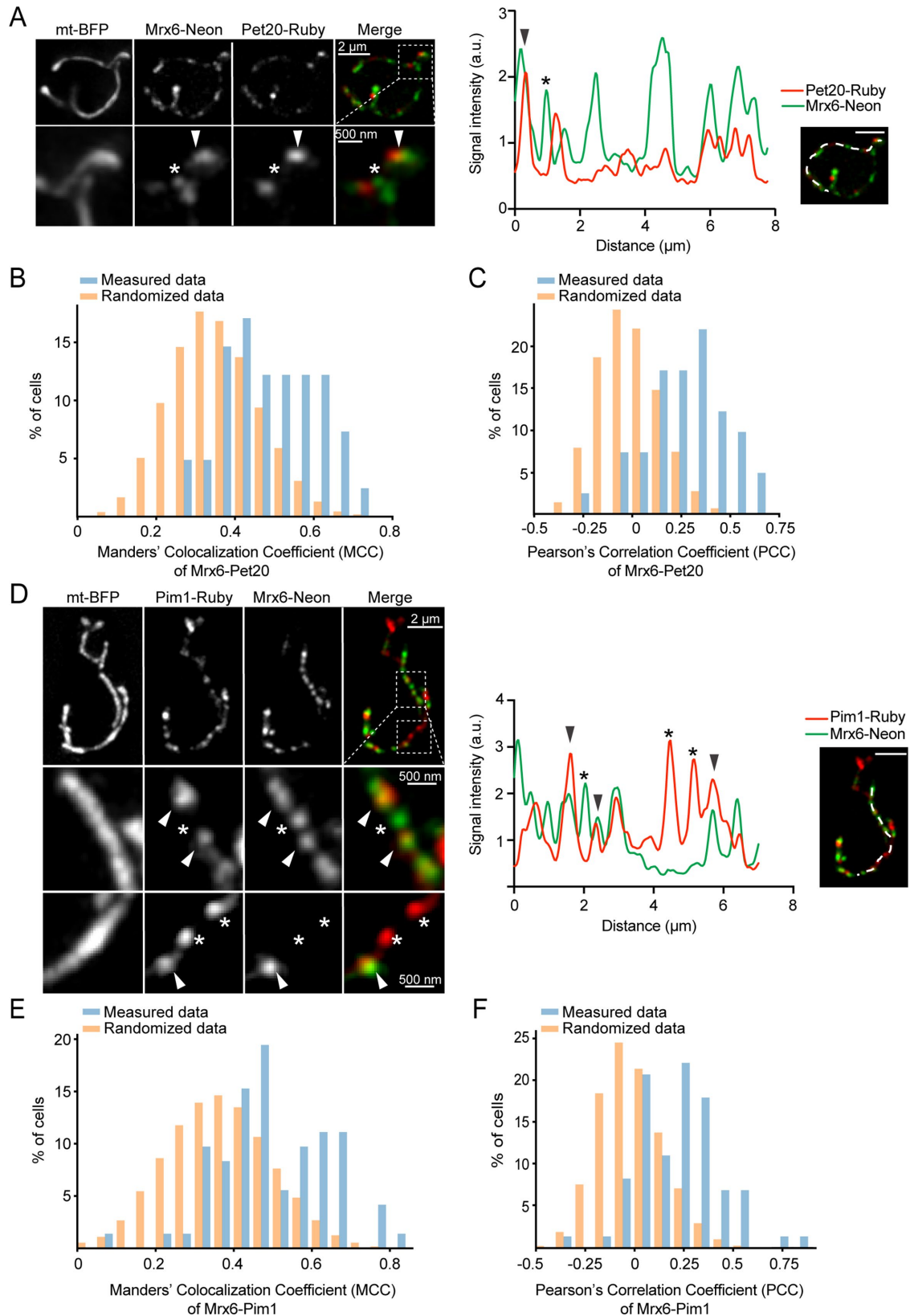
In summary, we propose the model in Figure 11: *Mrx6* in complex with *Pim1*, *Pet20*, and *Mam33* localizes to mtDNA. *Mrx6* then facilitates substrate recognition of *Pim1* and degradation of factors that stimulate mtDNA replication. Accordingly, absence of *Mrx6* would lead to an accumulation of such factors and in turn increase the number of mtDNA in the organelle.

Mitochondria evolved as endosymbionts from ancestral bacteria. In this light, it is exciting that Lon proteases are also involved in regulating replication of bacterial genomes. *Caulobacter crescentus* and *Escherichia coli* Lon proteases, for example, affect DNA replication by degrading a replication initiation factor and an inhibitor, respectively (Langklotz and Narberhaus, 2011; Jonas *et al.*, 2013). Therefore, our results identify a novel component in an evolutionarily conserved regulatory mechanism of mtDNA replication.

## MATERIALS AND METHODS

### Yeast strains and plasmids

The yeast strains used in this study are derived from W303 and are listed in Supplemental Table 2. Deletion of yeast genes was performed in diploid strains and C-terminal tagging of genes was done in haploid strains using homologous recombination as described previously (Janke *et al.*, 2004). Haploid cells were used for all experiments, except those shown in Figures 2A, 3, and 4, where diploids



**FIGURE 8:** Mrx6 partially colocalizes with Pet20 and Pim1. (A) Z-projections of microscopic images of a live cell expressing Mrx6-Neon (green) and Pet20-Ruby (red). Linescan analysis of mitochondrial network is shown on the right. Arrowhead indicates colocalization of Mrx6-Neon and Pet20-Ruby; asterisk marks noncolocalization. Cells were grown in SD-ura-trp. (B) Distribution of MCC determined for colocalization of Mrx6-Neon with Pet20-Ruby in measured ( $n = 41$  cells, mean = 0.50) and randomized data (mean = 0.36,  $p < 0.001$ ). (C) Distribution of PCC between Mrx6-Neon and Pet20-Ruby in measured ( $n = 41$  cells, mean = 0.28) and randomized data (mean = 0,  $p < 0.001$ ). (D) Z-projections of

cells were used. Plasmids and oligonucleotides used in this study are listed in Supplemental Tables 3 and 4, respectively.

### Colony blot hybridization

A previously described protocol (Kleinman, 1996) was followed with minor additions to optimize the protocol for fluorescent hybridization. Briefly, the mutants of the yeast deletion library were grown on glucose rich agar plates and transferred to nylon membranes (Bright Star-plus was used for the first and second screens, and Pall Biodyne A was used for the third screen) by incubating membranes on plates for 5 min, followed by gentle lifting. Membranes were air-dried for 5 min; placed, colony side up, on Whatman 3M papers that were soaked with reducing buffer (1 M sorbitol, 50 mM DTT, 20 mM EDTA, 10 mM NaAzide, 10 mM KF); and kept at RT (room temperature) for 20 min. Subsequently, membranes were transferred onto Whatman 3M papers that were soaked with lysis buffer (1 M sorbitol, 10 mM DTT, 20 mM EDTA, 10 mM Tris-HCl, pH 7.6, 10 mM NaAzide, 10 mM KF, 3 mg/ml zymolyase 20-T) and kept overnight in a closed container at 37°C. The next day, membranes were placed on Whatman 3M papers that were soaked with 0.5 M NaOH for 10 min. Membranes were air-dried for 5 min and neutralized by incubating on Whatman 3M papers saturated with 0.5 M Tris-HCl, pH 7.5/5X SSC (saline sodium citrate) for 5 min, two times, and transferred onto Whatman 3M papers saturated with TE pH 7.5/1X SSC (150 mM NaCl, 15 mM sodium citrate) for 5 min. Following neutralization, membranes were placed on Whatman 3M papers soaked with TE pH 7.5/1X SSC buffer with 0.2 mg/ml RNaseA (Sigma) and kept in a closed container for 2 h at 37°C. Subsequently, membranes were placed on Whatman 3M papers that were soaked with 100 mM Tris-HCl, pH 7.5/1X SSC for 5 min two times, air-dried, and baked at 65°C for 30 min, followed by cross-linking with 254-nm UV irradiation at 60 mJ/cm<sup>2</sup>. Membranes were placed in hybridization bottles and washed two times with 5X SSC, 0.5% SDS, 10 mM EDTA for 15 min while rotating at 65°C. Membranes were rinsed with Proteinase K buffer (50 mM Tris HCl, pH 7.5, 10 mM EDTA, 1% SDS, 50 mM NaCl) and incubated in Proteinase K buffer containing 2 mg/ml Proteinase K (Invitrogen) for 2 h at 55°C. Membranes were rinsed with 5X SSC and washed with 3 M urea, 1% SDS at 55°C, three times for 10 min each. Membranes were further washed with 5X SSC two times for 15 min each and prehybridized for 2 h with hybridization buffer (50% formamide, 8% dextran sulfate, 2.5X SSC, 5 mM EDTA, 25 mM HEPES-KOH, pH 7, 3% SDS) at 42°C. Membranes were hybridized with fluorescently labeled probes (final concentration 100 ng/ml mtDNA-Cy3 probe mix and 100 ng/ml nuclear DNA-Cy5 probe mix) in hybridization buffer at 42°C overnight. The next day, membranes were washed with wash buffer (1X SSC, 1% SDS) three times for 10 min each at 65°C. Membranes were completely air-dried before being scanned with a Typhoon fluorescence scanner using Cy3 and Cy5 channels at normal sensitivity.

### Preparation of probes for hybridization

Probes were prepared by PCR using Phusion DNA polymerase and different pairs of primers (Supplemental Table 4). Twelve different probes were pooled to detect nuclear DNA, and two different

probes were pooled to detect mtDNA. PCR products were cleaned and concentrated with Zymo DNA Clean & Concentrator-5 and labeled with Mirus Label IT nucleic acid labeling kits using Cy3 or Cy5 dyes overnight according to the product manual. Labeled probes were EtOH-precipitated and stored at -30°C. Probes were boiled for 5 min before addition into hybridization buffer.

### Quantification of colony blots

Scans of colony blots were quantified with ImageJ. The median signal intensity of each colony, for Cy3 and Cy5 channels, was determined after background subtraction using a rolling ball plug-in. Autofluorescence of yeast colonies was measured in both channels from a sample membrane that had not been incubated with probes. Autofluorescence of colonies correlated linearly with colony size, and thus we developed an algorithm that calculates autofluorescence for each mutant depending on its colony size. The hybridization signal for each colony was calculated by subtracting the estimated autofluorescence from the median signal intensity. However, later we found out that autofluorescence also correlates with respiratory capability, which explains why the mutants that lack mtDNA have mtDNA/nDNA ratios below zero after subtraction of colony autofluorescence. To calculate relative fold changes in mtDNA/nDNA ratios, mtDNA/nDNA ratios of all mutants except the ones that had lost mtDNA were averaged and used for normalization of each mutant mtDNA/nDNA ratio.

### Cell growth and quantitative PCR

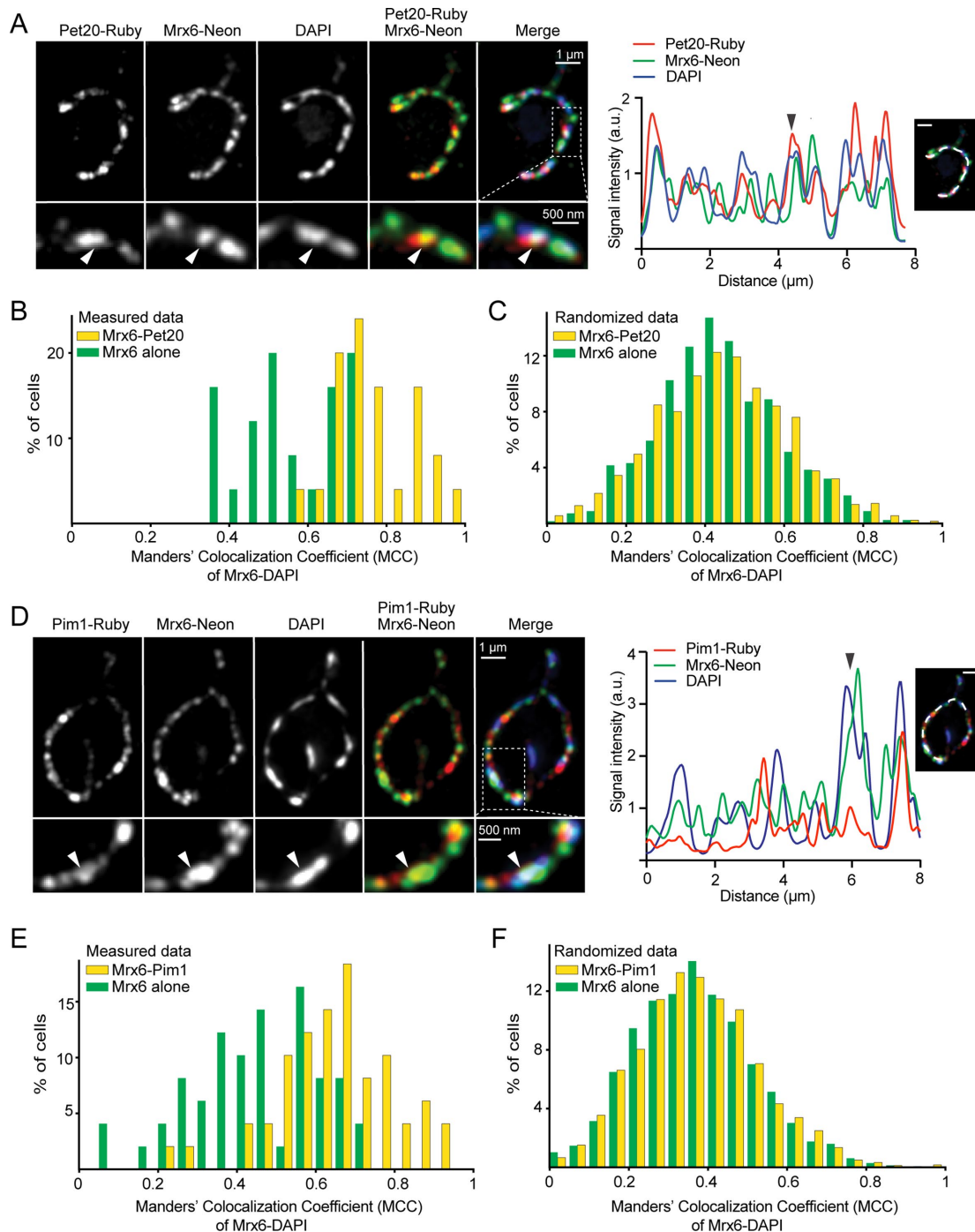
Prior to harvesting, yeast cells were grown in liquid media (YPD [yeast extract peptone dextrose], YPEG [yeast extract peptone ethanol glycerol], or drop-out synthetic media) in log phase for 24 h at 30°C. In Figure 2C, cells were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> in YPD for 1 h. In Figure 2D, cells were treated with DMSO (dimethyl sulfoxide) or FCCP (5 µg/ml) in YPD for 1 h. Genomic DNA (gDNA) was extracted using the Thermo Scientific Pierce Yeast DNA extraction rReagent or Zymo ZR-96 fungal/bacterial DNA kits. gDNA was subjected to qPCR using iQ-Syber Green Supermix (Bio-RAD) and primers specific for Cox1 and Act1 genes according to the manufacturer's manual (Supplemental Table 4). For absolute mtDNA copy number quantification, 1-kb fragments of Cox1 and Act1 genes were cloned into pUC19 plasmids and used as standards for copy number quantification. For statistical analysis of qPCR data, an unpaired *t* test was used for comparison of two groups and one-way analysis of variance was used for multiple comparisons, followed by Tukey's multiple comparison test in GraphPad Prism.

### Flow cytometry

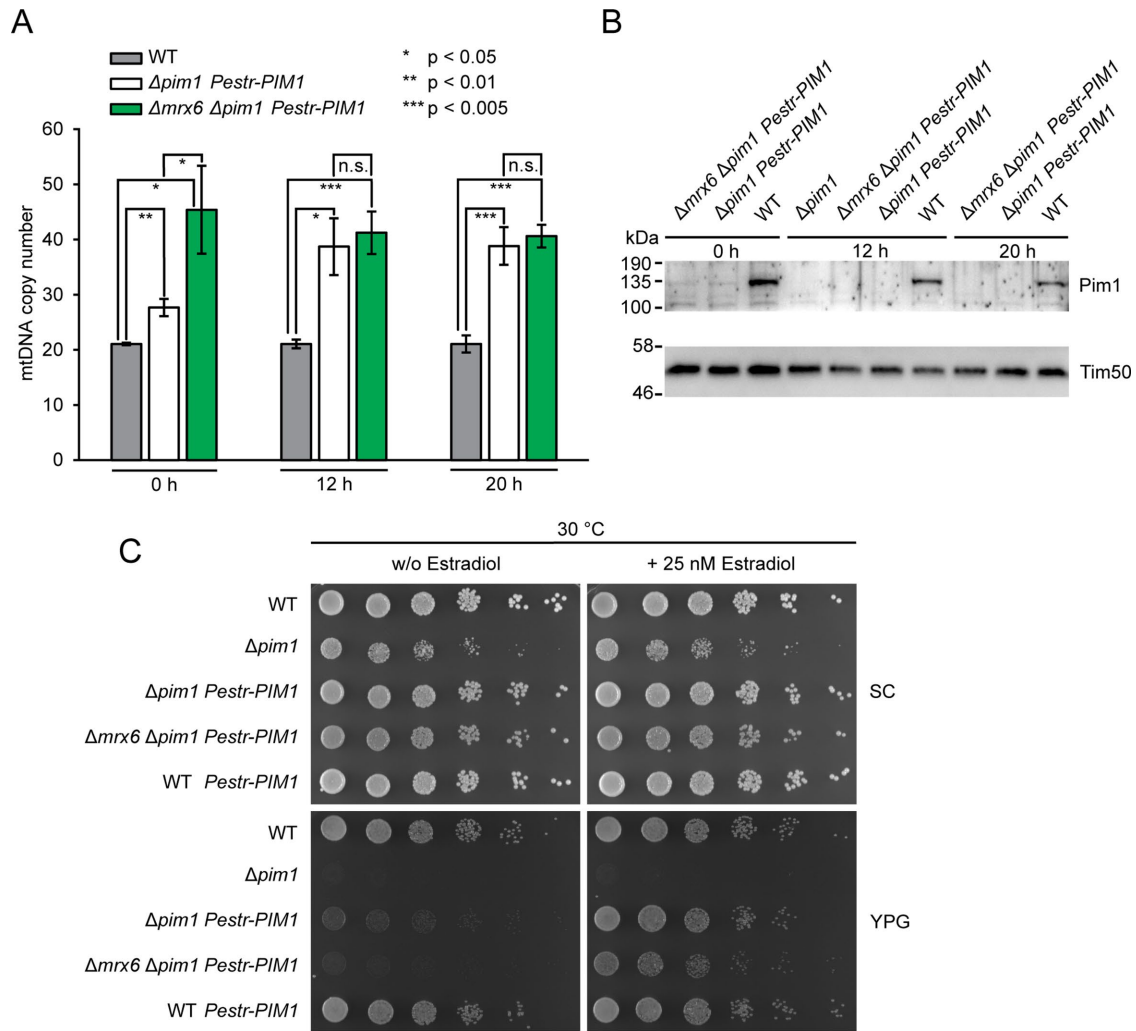
Yeast cells were grown overnight in liquid media (YPD) in 96-well polystyrene plates at 30°C and the next morning were diluted, re-grown for ~4 doubling times, and harvested at OD<sub>600</sub> = ~0.5–1. Yeast cultures were then transferred to 96-well microplates (Corning), diluted with YPD at a 1:5 ratio, and analyzed by a flow cytometer (LSR II, Beckton–Dickinson) and a high-throughput sampler (BD high-throughput sampler; Beckton–Dickinson) to inject samples into

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microscopic images of a live cell expressing Mrx6-Neon (green) and Pim1-Ruby (red). Linescan analysis of mitochondrial network is shown on the right. Arrowhead indicates colocalization of Mrx6-Neon and Pim1-Ruby; asterisk marks noncolocalization. Cells were grown in SD-ura-trp. (E) Distribution of MCC determined for colocalization of Mrx6-Neon with Pim1-Ruby in measured ( $n = 69$  cells, mean = 0.50) and randomized data (mean = 0.37,  $p < 0.001$ ). (F) Distribution of PCC between Mrx6-Neon and Pim1-Ruby in measured ( $n = 69$  cells, mean = 0.22) and randomized data (mean = 0,  $p < 0.001$ ).



**FIGURE 9:** Mrx6 colocalizes with Pet20 and Pim1 in regions close to mtDNA. (A) Z-projections of microscopic images of a live cell expressing Mrx6-Neon and Pet20-Ruby. mtDNA was stained with DAPI. Mrx6-Neon (green), Pet20-Ruby (red), and DAPI (blue) in the merged images. Linescan analysis of mitochondrial network is shown on the right. Arrowhead indicates colocalization of Mrx6-Neon, Pet20-Ruby, and DAPI signal. Cells were grown in SD-trp. (B) Distribution of MCC determined for colocalization of Mrx6-Pet20 (Mrx6-Neon colocalizing with Pet20-Ruby) with DAPI signal ( $n = 25$  cells, mean = 0.77) and Mrx6 alone (not colocalizing with Pet20-Ruby) with DAPI signal (mean = 0.56,  $p < 0.001$ ). (C) Distribution of MCC determined for colocalization of Mrx6-Pet20 (Mrx6-Neon colocalizing with Pet20-Ruby) with randomized DAPI signal ( $n = 25$  cells, mean = 0.44) and Mrx6-Neon alone (not colocalizing with Pet20-Ruby) with randomized DAPI signal (mean = 0.45,  $p = 0.78$ ). (D) Z-projections of microscopic images of a live cell expressing Mrx6-Neon (green) and Pim1-Ruby (red). mtDNA was stained with DAPI (blue). Linescan analysis of mitochondrial network is shown on the right. Arrowhead indicates colocalization of Mrx6-Neon, Pim1-Ruby, and DAPI signal. Cells were grown in SD-trp. (E) Distribution of MCC determined for colocalization of Mrx6-Pim1 (Mrx6-Neon colocalizing with Pim1-Ruby) with DAPI signal ( $n = 49$  cells, mean = 0.65) and Mrx6 alone (not colocalizing with Pim1-Ruby) with DAPI signal (mean = 0.46,  $p < 0.001$ ). (F) Distribution of MCC determined for colocalization of Mrx6-Pim1 (Mrx6-Neon colocalizing with Pim1-Ruby) with randomized DAPI signal ( $n = 49$  cells, mean = 0.38) and Mrx6 alone (not colocalizing with Pim1-Ruby) with randomized DAPI signal (mean = 0.37,  $p = 0.49$ ).



**FIGURE 10:** Acute depletion of Pim1 results in increased mtDNA copy number similar to levels of  $\Delta mrx6$  cells, but does not further exceed these levels in the double mutant. (A) qPCR analyses of mtDNA copy number in cells that harbor an estradiol-inducible Pim1 expression construct and lack either only *PIM1* ( $\Delta pim1$  Pestr-PIM1) or both *PIM1* and *MRX6* ( $\Delta mrx6 \Delta pim1$  Pestr-PIM1). Estradiol (25 nM) was supplied for Pim1 expression and omitting estradiol from the medium induced acute depletion of Pim1. Cells were continuously maintained at log phase in SC medium and harvested at  $OD_{600} = 1$  at the respective time points for DNA extraction ( $n = 3$  with three technical replicates each). (B) Western blot analyses of Pim1 levels in estradiol-regulatable strains and WT. The cells harvested for A were used for this experiment as well. (C) Drop dilution growth analysis of estradiol-inducible Pim1 strains in comparison to WT and  $\Delta pim1$ . The 11,200 cells were serially diluted at a ratio of 1:5 and the dilutions were spotted on fermentable (SC-medium) and nonfermentable carbon sources (YPG-medium) supplemented with 25 nM estradiol or not, and cultivated for 2 d at 30°C.

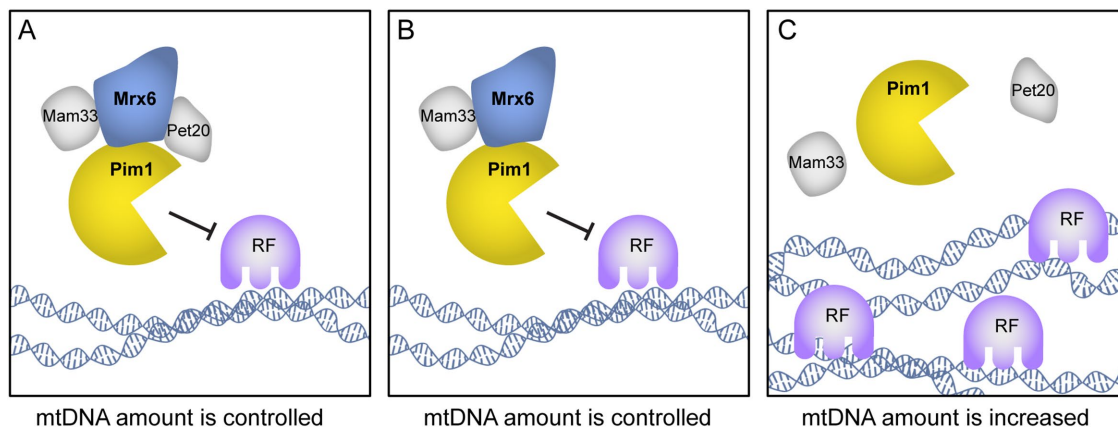
the flow cytometer. The SSC-H parameter was used as an estimate of cell size, and the SSC-H values of the mutants were normalized to the value of WT. Cell size of some mutants was also analyzed by microscopy to verify cell size increase. The remaining cultures were used for gDNA isolation and subjected to qPCR for mtDNA analysis for Figure 1D.

### Growth analysis

Yeast cells were grown in liquid media (YPD) in log phase for 24 h at 30°C and diluted to  $OD_{600} = 0.05$  in total 100  $\mu$ l YPD or diluted to  $OD_{600} = 0.1$  in a total of 100  $\mu$ l YPEG in a 96-well clear-bottomed microplate (Corning). The wells at the edges of the plate were filled with YPD to maintain humidity and the lid was secured using a tape that partially covered the plate to allow air exchange. Growth assays were conducted at 30°C using a Tecan Infinite 200 Pro plate reader for 48 h with a kinetic interval of 15 min.

### Immunoprecipitation

Immunoprecipitations were performed as previously described (Friedman *et al.*, 2015). Briefly, 500  $OD_{600}$  cells grown in log phase in YPD at 30°C were harvested by centrifugation and resuspended in 5 ml of lysis buffer (20 mM HEPES, pH 7.4, 150 mM KOAc, 2 mM Mg(OAc)<sub>2</sub>, 1 mM EGTA [ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 0.6 M sorbitol), and protease inhibitor cocktail was added to 1x (EDTA-free; Roche). Cell suspension was flash-frozen dropwise in liquid N<sub>2</sub> and lysed using a ball mill (Retsch MM301). The cell powder was thawed at RT, and unbroken cells and large debris were pelleted using a GH-3.8 rotor at 1500 rpm for 5 min at 4°C. For solubilization, digitonin was added to the supernatant to a final concentration of 1%. Samples were incubated for 30 min at 4°C and cleared by centrifugation at 12,000  $\times$  g at 4°C.  $\mu$ MACS anti-Flag beads (Miltenyi Biotec; 50  $\mu$ l) were added to the supernatant and incubated for 45 min at 4°C, followed by isolation



**FIGURE 11:** Model for the role of the Mrx6 complex in mtDNA replication. (A) The Mrx6 complex colocalizes preferentially with mtDNA, whereas single components are more often found in areas that lack mtDNA. (B) Mrx6 is essential for the formation of a Pim1-containing complex that controls mtDNA levels, whereas Pet20 is dispensable for sufficient mtDNA copy number control. (C) We propose that Mrx6 may facilitate substrate recognition of Pim1 and degradation of replication factors (RF) that stimulate mtDNA replication. Accordingly, absence of Mrx6 would lead to accumulation of such factors and result in increased amounts of mtDNA.

with  $\mu$  columns and a  $\mu$ MACS separator (Miltenyi Biotec). Columns were washed three times with lysis buffer, 0.1% digitonin, and 1x protease inhibitor and two times with only lysis buffer. Samples were eluted using on-bead trypsin digest by incubating beads with 25  $\mu$ l of elution buffer I (2M urea, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 5  $\mu$ g/ml trypsin [Trypsin Gold, Promega]) for 30 min at RT. Elution buffer II (50  $\mu$ l; 2 M urea, 50 mM Tris-HCl, pH 7.5, 5 mM chloroacetamide) was added to the column two times to collect elutions. Elutions were kept at RT overnight to continue digestion. Mass spectrometric proteomic analysis was performed at the Genome Center Proteomics Core of the University of California, Davis.

### Western blot analysis

For Figure 7A, the samples eluted from the  $\mu$ MACS beads by incubating beads with preheated (95°C) 1x SDS loading buffer instead of trypsin digestion. For Figure 2A and Supplemental Figure 8, proteins were extracted from 1  $OD_{600}$  cells in urea-CHAPS buffer (20 mM Tris-HCl, pH 7.4, 7 M urea, 2 M thiourea, 4% CHAPS) by 3 min bead beating at 4°C. The protein concentration of each sample was measured by Pierce BCA protein assay kit (Thermo Scientific). Samples were boiled for 5 min in SDS loading dye and BME ( $\beta$ -mercaptoethanol) before SDS-PAGE analysis, transferred to nitrocellulose membrane, and immuno-blotted with the following primary antibodies at the indicated concentrations in 5% milk PBS-T (phosphate-buffered saline, 0.1% Tween 20)/TBS-T (Tris-buffered saline, 0.1% Tween 20) buffer: mouse anti-FLAG M2 (1:5000; Sigma-Aldrich); rabbit anti-Pim1 (1:1000; kindly provided by C. Suzuki, New Jersey Medical School, Rutgers, The State University of New Jersey); mouse anti-myc 9E10 (1:1000; Santa Cruz); mouse anti-PGK1 (1:5000; abcam); rabbit anti-Tom40 (1:30,000; kindly provided by T. Langer, Max-Planck-Institute for Biology of Ageing, Cologne, Germany); rabbit anti-Abf2 (1:1000; kindly provided by J. Nunnari, Department of Molecular and Cellular Biology, University of California, Davis, Davis, CA); rabbit anti-Tim50 (Mokranjac *et al.*, 2003) (1:1000; kindly provided by K. Hell, Biomedical Center Munich-Physiological Chemistry, Ludwig-Maximilians University, Planegg, Martinsried, Germany).

### Structured illumination microscopy and analysis

For Figure 3A, slides were prepared as previously described (Kaplan and Ewers, 2015). Briefly, microscope coverslips (High Precision)

were cleaned with a plasma cleaner (PDC-001, Harrick Plasma) and treated with concanavalin A (Sigma; 5 mg/ml) for 30 min, spin-coated for 15 s, and air-dried for 15 min in a vacuum desiccator. Before imaging, yeast cells were grown in liquid dropout synthetic media in log phase for 24 h at 30°C. A sample of 0.5  $OD_{600}$  cells was spun down, washed, and resuspended in 100  $\mu$ l PBS. A cell suspension (20  $\mu$ l) was added on the concanavalin A-treated coverslips and incubated for 15 min. Unattached cells were washed with PBS. For fixation, cells were incubated twice for 5 min with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS on coverslips. Fixation was followed by quenching two times with 50 mM  $NH_4Cl$ , 10 min each. Cells were washed with PBS and a drop of anti-fade medium (Vectashield) was added before mounting coverslips to slides. Slides were imaged using DeltaVision OMX SR (GE) using SIM mode and a 60x/1.42 NA oil objective. The Imaris software was used to detect/count LacI-GFP foci and to segment the mitochondrial network in three dimensions. Quantification of mitochondrial network length, mitochondrial endpoints, and distribution of mtDNA was performed as described previously (Osman *et al.*, 2015). Three- or four-way junctions of the segmented mitochondrial network were scored as branchpoints.

For Figure 4A, yeast cells were grown in liquid dropout synthetic media in log phase for 24 h at 30°C and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in growth media for 30 min at RT. Cells were prepared as described previously (Silver, 2009). Briefly, cells were pelleted and washed two times with P solution (0.1 M  $KH_2PO_4$ , 1.2 M sorbitol) and resuspended in 1 ml of P solution. After 15  $\mu$ l of 10 mg/ml zymolyase (T-20) and 5  $\mu$ l BME were added to the solution it was incubated at RT for 30 min. Cells were gently washed once with P solution and resuspended in 0.5 ml of P solution. Microscope coverslips (High Precision) were plasma-cleaned and coated with 0.1% poly-L-lysine for 20 min. Coverslips were washed two times with ddH<sub>2</sub>O and air-dried completely. Cell suspension (30  $\mu$ l) was added and incubated 20 min. Excess media were aspirated and coverslips were plunged into ice-cold methanol for 6 min, followed by submergence into ice-cold acetone for 30 s. Coverslips were air-dried briefly and incubated with 3% bovine serum albumin (BSA) (Sigma) in PBS for 1 h at RT. Cells were stained with DAPI (Invitrogen; 5  $\mu$ g/ml) for 5 min and washed two times with PBS. Coverslips were mounted on





number was determined by quantitative PCR using iQ-Syber Green Supermix (Bio-RAD) as described in *Cell growth and quantitative PCR*.

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