Mgr3p and Mgr1p Are Adaptors for the Mitochondrial i-AAA Protease Complex

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By screening yeast knockouts for their dependence upon the mitochondrial genome, we identified Mgr3p, a protein that associates with the i-AAA protease complex in the mitochondrial inner membrane. Mgr3p and Mgr1p, another i-AAA-interacting protein, form a subcomplex that bind to the i-AAA subunit Yme1p. We find that loss of Mgr3p, like the lack of Mgr1p, reduces proteolysis by Yme1p. Mgr3p and Mgr1p can bind substrate even in the absence of Yme1p, and both proteins are needed for maximal binding of an unfolded substrate by the i-AAA complex. We speculate that Mgr3p and Mgr1p function in an adaptor complex that targets substrates to the i-AAA protease for degradation.

INTRODUCTION

Mitochondria, found in virtually all eukaryotic cells, play key roles in a wide range of cellular processes, including ATP synthesis, ion homeostasis, amino acid biosynthesis, fatty acid metabolism, and apoptosis (Tzagoloff, 1982). Proteomic studies have shown that mitochondria contain >1000 proteins (Sickmann *et al.*, 2003; Jensen *et al.*, 2004; Prokisch *et al.*, 2004; Zahedi *et al.*, 2006), with the majority encoded on nuclear genes. These nuclear-encoded genes are translated in the cytosol and then sorted to the outer membrane (OM), intermembrane space (IMS), inner membrane (IM), or mitochondrial matrix (Koehler, 2004; Rehling *et al.*, 2004). In contrast, only a small fraction of mitochondrial proteins are encoded in the mitochondrial genome (mtDNA) (Attardi and Schatz, 1988). Most of these mtDNA-encoded proteins are subunits of IM complexes required for oxidative phosphorylation.

Although mtDNA is required for the viability of most eukaryotes, the yeast *Saccharomyces cerevisiae* can live in the absence of its mitochondrial genome (Tzagoloff, 1982; Chen and Clark-Walker, 2000). These “rho⁰” or “cytoplasmic petite” mutants lack essential subunits of the electron transport chain and ATP synthase complexes, generating cellular ATP only by glycolysis. Several proteins have been identified that are needed for yeast growth in the absence of mtDNA, even when cells are grown on fermentable medium. Yeast cells lacking these proteins are called “petite-negative” mutants (Chen and Clark-Walker, 2000). Without mtDNA, the electron transport chain is not functional; consequently, the generation of an inner membrane potential ($\Delta\Psi$) requires both the F₁ portion of the ATP synthase and the ATP/ADP antiporter (Dupont *et al.*, 1985; Giraud and Velours, 1997;

Chen and Clark-Walker, 1999). The exchange via the antiporter of cytosolic ATP⁻⁴ for ADP⁻³ produced by the matrix-localized F₁-ATPase is thought to generate the electrical gradient in petite cells. Because $\Delta\Psi$ is vital for essential mitochondrial functions besides oxidative phosphorylation, cells lacking subunits of the F₁-ATPase or the ATP/ADP antiporter are petite-negative.

The i-AAA complex, a mitochondrial protease located in the IM, is also crucial for mtDNA-independent growth (Thorsness *et al.*, 1993). The i-AAA protease is thought to consist of a complex of Yme1p subunits. Yme1p is a member of the AAA⁺ superfamily (ATPases associated with various cellular activities), which are typically organized into large, hexameric ring structures and use the energy from ATP hydrolysis to unfold substrates and translocate them into the narrow axial channel of the complex for degradation (Baker and Sauer, 2006). Yme1p is anchored in the IM with its proteolytic domain facing the IMS (Leonhard *et al.*, 1996). Although a few substrates for the i-AAA protease are known (Leonhard *et al.*, 1996, 1999, 2000; Augustin *et al.*, 2005), it is not understood why Yme1p-dependent turnover is needed for yeast viability in the absence of mtDNA. Recently, in a genome-wide screen for new petite-negative mutants, we identified Mgr1p (Dunn *et al.*, 2006). Mgr1p associates with Yme1p and is required for full activity of the i-AAA protease, but its role in mitochondrial quality control is unclear. In a survey of yeast knockouts lacking uncharacterized mitochondrial proteins, we now identify a new protein that we call Mgr3p. We find that Mgr3p and Mgr1p function together in an adaptor complex that seems to help target substrates to the i-AAA protease.

MATERIALS AND METHODS

Yeast Strains

Strains and an outline for their construction are listed in Supplemental Table S6. Oligonucleotide primers used for polymerase chain reaction (PCR)-mediated gene disruption (Sikorski and Hieter, 1989; Brachmann *et al.*, 1998) and protein tagging (Longtine *et al.*, 1998) are listed in Supplemental Table S7. As indicated, when diploid strains were the recipient of PCR-mediated disruption or tagging, sporulation of the diploid and isolation of meiotic segregants were used to generate haploid constructs. Yeast media and molecular genetic techniques were as described previously (Adams *et al.*, 1997).

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Plasmid Construction

The 2 μ -MGR3 plasmid pM527 was created by PCR amplification of the MGR3 gene by using primers 1867 and 1868 (Supplemental Table S7), followed by product digestion with XhoI and NotI and ligation into XhoI/NotI-cut pRS426 (Sikorski and Hieter, 1989).

Screening Knockouts for mtDNA Dependence

Wild-type cells and 42 yeast knockout strains (Supplemental Table S1) were grown overnight in YEPD medium containing 25 μ g/ml ethidium bromide (EtBr) and then tested for growth on YEPD medium without EtBr. One mutant, *yer038w-a* Δ , grew moderately better in EtBr-containing medium than the other strains tested. Interestingly, this gene disruption also removes YER039C, an overlapping gene of unknown function, but this strain was not analyzed further. Four knockouts, *yor286w* Δ , *ykr016w* Δ , *yer182w* Δ , and *ymr115w* Δ , showed reduced growth after EtBr treatment in comparison with wild type.

Mitochondrial Fractionation

Mitochondria were isolated from yeast cells as described previously (Daum *et al.*, 1982), except that SEH buffer (250 mM sucrose, 1 mM EDTA, and 20 mM HEPES-KOH, pH 7.4) with a 1:1000 dilution of protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO) was used. Osmotic shock of mitochondria and protease treatment of mitochondria and mitoplasts were as described previously (Kerscher *et al.*, 2000), except that 200 μ g of mitochondria or mitoplasts at 0.2 mg/ml were used. The separation of OM and IM was as described previously (Pon *et al.*, 1989). Treatment of mitochondria with 0.1 M Na₂CO₃ was as described previously (Kerscher *et al.*, 2000), except that samples were centrifuged at 100,000 $\times$ g for 60 min.

Analysis of i-AAA Complex

Blue native (BN) electrophoresis was performed as described previously (Dunn *et al.*, 2006). For precipitation of Mgr3p-His6, 500 μ g of mitochondria was solubilized in 500 μ l of extraction buffer (150 mM potassium acetate, 30 mM HEPES-KOH, 10 mM imidazole, 1% digitonin, and 1 $\times$ protease inhibitor cocktail) on ice for 15 min. Then, 500 μ l of extraction buffer lacking detergent was added to samples, and insoluble material was removed by centrifugation (13,000 $\times$ g for 10 min). Fifty to 100 μ l of nickel-nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN, VALENCIA, CA) was added to the cleared lysate, and samples were rotated for 1 h at 4°C. After removing 100 μ l (10% load), Ni-NTA beads were collected by centrifugation. After three washes in buffer containing 0.1% digitonin, 2 $\times$ SDS-sample buffer with 400 mM imidazole was added to beads and to the 10% load sample before polyacrylamide gel electrophoresis (PAGE) analysis. Precipitation of myc-tagged proteins from digitonin-solubilized mitochondria was as described previously (Dunn *et al.*, 2006).

Substrate Degradation Assays

Degradation of Yta10p(161)DHFR^{MUT} and Nde1p-HA was assayed as described in Dunn *et al.* (2006).

Substrate Binding Assays

³⁵S-labeled Yta10(161)-DHFR^{MUT} was imported into isolated mitochondria in the presence of ATP (but lacking an ATP regeneration system) at 25°C for 12 min. Import reactions were stopped on ice, and mitochondria were reisolated by centrifugation at 13,000 $\times$ g for 10 min through a sucrose cushion. Mitochondria were then resuspended at 1 mg/ml in 400 μ l of import buffer lacking ATP and NADH and incubated for 30 min at 31°C. To precipitate Mgr1p-myc along with bound substrate, mitochondria were pelleted by centrifugation (13,000 $\times$ g for 10 min) and resuspended in 500 μ l in lysis buffer (1% digitonin, 50 mM NaCl, 30 mM HEPES-KOH, pH 7.4, and 1:1000 dilution of protease inhibitor cocktail) on ice for 15 min. After centrifugation at 13,000 $\times$ g for 10 min to remove insoluble material, another 500 μ l of lysis buffer lacking detergent was added to the samples, followed by 75 μ l of a 50% slurry of anti-myc antibodies coupled to agarose beads. After mixing at 4°C for 4 h, 100 μ l were removed (10% total), and beads were washed three times with lysis buffer containing 0.1% digitonin and collected by centrifugation. Samples were analyzed by SDS-PAGE and phosphorimaging. Anti-myc antibodies were coupled to beads using a Seize primary immunoprecipitation kit (Pierce Chemical, Rockford, IL). Alternatively, antibodies were cross-linked to protein G-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) as described previously (Harlow and Lane, 1999), with the following modifications: Protein G-Sepharose beads (1 ml; settled) were washed and equilibrated in binding buffer (20 mM sodium phosphate, pH 7.0) before mixing with 1 ml of anti-myc ascites fluid at 4°C overnight. After pelleting, myc-beads were resuspended in 1.5 ml of binding buffer containing 14 mM disuccinimidyl suberate (DSS; Pierce Chemical) and incubated for 1 h at room temperature. After washing the myc-beads in binding buffer, the DSS was quenched with 2 ml of 0.1 M ethanolamine for 1 h at room temperature. Beads were washed with 10 ml of 0.1 M glycine, pH 2.8, and then four times with 10 ml of binding buffer.

To precipitate Mgr3p-His6 or Yme1p-His6 along with bound substrate, mitochondria were solubilized on ice in 500 μ l of lysis buffer containing 10 mM imidazole. After 15 min, 500 μ l of lysis buffer lacking detergent was added to samples and centrifuged at 13,000 $\times$ g for 10 min. Ni-NTA beads (~50–100 μ l) were added to the mitochondrial extract and mixed at 4°C for 1 h. After removal of 100 μ l of mix (10% total), beads were washed three times with wash buffer containing 10 mM imidazole, and proteins from the beads and loading control were extracted with 2 $\times$ SDS sample buffer containing 0.6 M β -mercaptoethanol and 400 mM imidazole.

Immunoblotting

Proteins were analyzed by SDS-PAGE and immunoblotting to Immobilon filters (Millipore, Billerica, MA) by using standard techniques (Current Protocols Online; <http://www.mrw2.interscience.wiley.com/cponline>). The myc-tagged proteins were visualized using mouse ascites fluid prepared using 9E10 cells (Covance, Denver, PA; Evan *et al.*, 1985). His6-tagged proteins were detected using rabbit polyclonal antiserum SC-803, purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mitochondrial proteins were identified using anti-serum to Tim23p (Emtage and Jensen, 1993), Mas2p (Jensen and Yaffe, 1988), Tom70p (Hase *et al.*, 1984), Yme1p (Thorsness *et al.*, 1993), OM45 (Yaffe *et al.*, 1989), or F₁F₀ (a gift from A. Davis, Tufts University School of Medicine, Boston, MA). Immune complexes were visualized using horseradish peroxidase-conjugated secondary antibody (GE Healthcare) followed by chemiluminescence (West Pico; Pierce Chemical). Immune blots were imaged using a Versadoc imaging system and Quantity One software (Bio-Rad, Hercules, CA) or by exposure to x-ray film.

RESULTS

Mgr3p Is Required for mtDNA-independent Yeast Growth

To identify new petite-negative mutants, we screened a subset of the *S. cerevisiae* knockout collection for strains unable to grow in the absence of mtDNA. We focused on a set of genes that recent studies have shown to encode mitochondrial proteins (Sickmann *et al.*, 2003; Zahedi *et al.*, 2006) and that were uncharacterized at the time we began our study. Forty-two mutant strains from the haploid yeast knockout collection (Giaever *et al.*, 2002) were grown overnight in medium containing EtBr to force mtDNA loss (Slonimski *et al.*, 1968; Goldring *et al.*, 1970) and tested for viability (Supplemental Table S1). Although the growth of 38 of the knockout strains was not inhibited by the treatment, as compared to wild-type, four of the mutants grew slowly or not at all after treatment with EtBr. Because the growth of the *ymr115w* Δ knockout was the most severely affected by EtBr treatment, we chose this strain for further analysis and named the gene Mitochondrial Genome Required (MGR3). We note that an earlier name for MGR3 was FMP24 (Sickmann *et al.*, 2003). *mgr3* Δ is inviable when loss of mtDNA is induced by EtBr, similar to *yme1* Δ and *mgr1* Δ , mutants that are deficient in i-AAA protease activity, and *atp2* Δ , which lacks F₁-ATPase function (Figure 1A). Furthermore, we confirmed that the lethality of *mgr3* Δ after EtBr treatment is due to the loss of mtDNA and is not due to other effects of EtBr treatment. In particular, *mgr3* Δ strains lacking mtDNA were inviable even in the absence of EtBr when they were forced to lose a plasmid carrying wild-type MGR3 (Figure 1B). We do note that the mtDNA dependence of *mgr3* Δ is background specific: *mgr3* Δ generated in an unrelated strain (Thorsness and Fox, 1993) is not petite negative and continues to grow after EtBr treatment (Figure 1C). This strain dependence was also seen for *mgr1* Δ mutants (Dunn *et al.*, 2006).

Mgr3p is predicted to encode a 58-kDa protein with a single transmembrane segment near its amino terminus (residues 78–99) and three potential coiled-coil regions (Supplemental Figure S2). Although Mgr3p is remarkably similar to another *S. cerevisiae* protein encoded by YKL133C (35% identical by BLASTP), *ykl133c* Δ knockouts are not petite negative (Dunn, unpublished data). Orthologues of Mgr3p exist in other yeast, such as *Ashbya gossypii* (AFR197W; 52% identi-

Figure 1. Mgr3p is required for mtDNA-independent growth. (A) Yeast cells lacking Mgr3p are petite-negative. Wild-type strain BY4742, *atp2Δ* strain RJ2092, *yme1Δ* strain RJ2051, *mgr1Δ* strain RJ2058, and *mgr3Δ* strain RJ2122 were grown for 23 h in YEPD medium containing 25 μg/ml ethidium bromide (+EtBr) or for 8 h in YEPD medium (−EtBr) at 30°C. Then, 10-fold serial dilutions were made from cultures at OD₆₀₀ of 0.02, and 4 μl of each dilution was spotted onto YEPD medium with or without EtBr. Plates were incubated for 2 d at 30°C. (B) *mgr3Δ* lethality upon EtBr treatment is due to mtDNA loss. Wild-type strain BY4742 and *mgr3Δ* strain RJ2122 were transformed with empty vector pRS426 or a 2μ plasmid carrying the *MGR3* gene. The resulting transformants were grown overnight in SD medium lacking uracil and then transferred to YEPD medium containing EtBr and grown for ~24 h at 30°C. Cells were diluted to OD₆₀₀ of 0.02 and 0.002, and 4 μl of each dilution were spotted to YEPD medium lacking EtBr and grown for a further 2 d at 30°C (YEPD). Next, the resulting rho⁰ WT and *mgr3Δ* strains containing the 2μ-MGR3 plasmid were grown in liquid medium for a further 1 d in YEPD medium and then diluted to OD₆₀₀ of 0.02. Four microliters of each dilution was spotted to complete medium containing 5-fluoroorotic acid and grown for a further 1 d at 30°C (5-FOA). (C) The petite-negative phenotype of *mgr3Δ* is background specific. The following strains were patched twice to YEPD medium containing 25 μg/ml EtBr and then struck to YEPD medium for 2 d at 30°C. In Winston strain background FY (Winston *et al.*, 1995): parental strain RJ1962 [WT], RJ1974 [*yme1Δ*], RJ1961 [*mgr1Δ*], RJ2150 [*mgr3Δ*], RJ2152 [*mgr1Δmgr3Δ*]; and in Thorsness strain background PTY: strain PTY33 [WT], strain RJ2001 [*mgr1Δ*], strain RJ2326 [*mgr3Δ*], and strain RJ2040 [*yme1Δ*].

cal), *Kluyveromyces lactis* (KLLA0B12419g; 52% identical), and *Candida albicans* (CaO19.11835; 32% identical), but their

function in these different organisms is not known. We do not find significant homology between the amino acid sequence of Mgr3p and any nonfungal proteins.

Mgr3p Is a Mitochondrial Inner Membrane Protein

Mgr3 protein was previously found in the proteome of highly purified yeast mitochondria (Sickmann *et al.*, 2003) and also localized to mitochondria during a genome-wide survey of green fluorescent protein fusion protein localization (Huh *et al.*, 2003). To confirm this location, we fused the myc epitope to the carboxy terminus of Mgr3p and monitored the fully functional Mgr3p-myc protein during subcellular fractionation. Cells expressing Mgr3p-myc were homogenized and separated into a mitochondrial fraction and a postmitochondrial supernatant. We found by immunoblotting that Mgr3p-myc cofractionated with the mitochondrial protein Tim23p (Emtage and Jensen, 1993) and not with hexokinase, a cytosolic marker (Kerscher *et al.*, 2000) (Figure 2A). When mitochondria were treated with alkali, both Mgr3p-myc and Tim23p, an integral IM protein (Emtage and Jensen, 1993), were found in the pellet fraction (Figure 2B). In contrast, Mas2p, a soluble matrix marker (Jensen and Yaffe, 1988), was released by the carbonate treatment. Our results indicate that Mgr3p is an integral membrane protein, consistent with the prediction of a transmembrane segment near its amino terminus. When mitochondria were sonicated and the resulting membrane vesicles separated on a sucrose gradient, Mgr3p-myc cofractionated with the IM marker, the β-subunit of the F₁-ATPase (F₁β), but not with the OM marker, OM45 (Yaffe *et al.*, 1989) (Figure 2C). Thus, Mgr3p is a mitochondrial inner membrane protein.

The carboxy terminus of Mgr3p faces the IMS. As shown in Figure 2D, when intact mitochondria were treated with protease, Mgr3p-myc was protected from digestion along with the inner membrane protein Tim23p and matrix-localized Mas2p. Only Tom70p, which is exposed to cytosol (Hase *et al.*, 1984), was digested by the protease treatment. However, when the OM was ruptured by osmotic shock (OS) to form mitoplasts, the immunoreactive amino terminus of Tim23p (Ryan *et al.*, 1998) and the myc tag of Mgr3p-myc were both digested, whereas matrix-localized Mas2p was protected. Because no protease-protected Mgr3p-myc fragments were detected after protease digestion of mitoplasts, we conclude that Mgr3p is inserted into the mitochondrial IM with a small amino-terminal segment exposed to the matrix and a large carboxy-terminal domain facing the IMS.

Mgr3p Associates with the i-AAA Protease

We find that Mgr3p coprecipitates from mitochondrial extracts with Yme1p and the i-AAA-associated Mgr1p protein. For these studies, we used a Mgr1p-myc fusion protein (Dunn *et al.*, 2006) and also engineered six histidine residues at the carboxy terminus of Mgr3p, forming a fully functional Mgr3p-His6 construct. Mitochondria were isolated from cells expressing both Mgr3p-His6 and Mgr1p-myc, solubilized in digitonin-containing buffer, and the Mgr3p-His6 protein precipitated with Ni-NTA beads. Both Yme1p and Mgr1p-myc coprecipitated with Mgr3p (Figure 3A, lane 4). Although nearly all of the Mgr3p-His6 protein was found in the pellet fraction, we note that only ~10% of Yme1p and Mgr1p-myc precipitated along with Mgr3p. Therefore, the binding of Mgr3p to Mgr1p and Yme1p may be weak or dynamic. Nonetheless, the Mgr3p-Mgr1p-Yme1p interaction is specific, because we did not see any coprecipitation when mitochondria from cells lacking the Mgr3p-His6 fu-

Figure 2. Mgr3p-myc is a mitochondrial inner membrane protein with its carboxy terminus exposed to the IMS. (A) Mgr3p is a mitochondrial protein. Strain RJ2165, which expresses Mgr3p-myc, was homogenized (H) and separated into a mitochondrial pellet fraction (M) and crude cytosolic supernatant fraction (S) by centrifugation. Cell equivalents were then subjected to immunoblotting and decorated with antibodies to hexokinase, Tim23p, or the myc epitope (Mgr3p-myc). (B) Mgr3p is an integral membrane protein. Mitochondria from strain RJ2165 were resuspended in buffer or 0.1 M Na₂CO₃. After centrifugation, equal amounts of the supernatant (S) or pellet (P) fractions were analyzed by immunoblotting using antibodies to Mas2p, Tim23p, or the myc epitope (Mgr3p-myc). (C) Mgr3p is associated with the inner membrane. Mitochondria containing Mgr3p-myc were sonicated and membrane vesicles separated on sucrose step gradients. Fractions were analyzed by immunoblotting with antibodies to OM45, F₁β, or the myc epitope (Mgr3p-myc). Fraction 2 represents the top of the gradient. (D) The carboxy terminus of Mgr3p-myc faces the IMS. Intact mitochondria, or mitochondria with their outer membranes disrupted by OS, were digested with 50 μg/ml proteinase K (PK) for 30 min, and then analyzed by immunoblotting using antibodies to Tom70p, Tim23p, Mas2p, or the myc epitope (Mgr3p-myc).

sion protein were used (Figure 3A, lane 2). In addition, similar amounts of Yme1p were found to coprecipitate with an Mgr3p-myc fusion protein (Dunn, unpublished data).

Blue native gel electrophoresis also indicates that Mgr3p associates with the i-AAA complex. Mitochondria were iso-

lated from cells expressing Yme1p, Mgr1p-myc, and Mgr3p-His6, solubilized in digitonin, and proteins separated on nondenaturing gels (Schagger and von Jagow, 1991). Most Mgr1p-myc comigrated with the larger of two resolvable Yme1p-containing complexes (Figure 3B, lanes 1 and 2, white arrow), both larger than 667 kDa, in agreement with previous findings (Dunn *et al.*, 2006). Supporting the association of Mgr3p with the i-AAA protease complex, Mgr3p-His6 was found to comigrate with Mgr1p-myc and Yme1p during BN-PAGE (Figure 3B, lane 3, white arrow). Further evidence that Mgr3p interacts with the i-AAA complex comes from examining the migration of the catalytic Yme1p subunit after deletion of MGR3. Mitochondria were isolated from wild-type cells, as well as from *mgr1Δ*, *mgr3Δ*, and *mgr1Δ mgr3Δ* mutants. As shown in Figure 3B, Yme1p in mitochondrial extracts from wild-type cells migrates in two complexes (Figure 3C, lanes 1 and 5; white and black arrows). Because the larger band disappeared and the smaller band increased in intensity in mitochondria lacking Mgr1p (Figure 3C, lane 2), Mgr3p (Figure 3C, lane 3), or both Mgr1p and Mgr3p (Figure 3C, lane 4), we conclude that the upper band consists of a supercomplex containing Mgr3p, Mgr1p, and the i-AAA protease. Supporting this view, most of Mgr1p (Figure 4B, lane 1, white arrow) migrates in a single, large band that is dependent upon the presence of Mgr3p and Yme1p (Figure 4B, lanes 2–4). Similar results were obtained when the mobility of Mgr3p was examined in the presence or absence of Mgr1p and Yme1p (Supplemental Figure S3).

Mgr3p and Mgr1p Form a Subcomplex

In the absence of Yme1p, we found that Mgr3p and Mgr1p remain associated. When Mgr3p-His6 was precipitated from digitonin-treated mitochondria, the same amount of the Mgr1p-myc fusion protein coprecipitated with Mgr3p-His6 when cells expressed Yme1p (Figure 4A, lane 2) or lacked Yme1p (Figure 4A, lane 4). Additional support for a Yme1p-independent interaction between Mgr1p and Mgr3p comes from blue native gel analyses. In mitochondria expressing both Yme1p and Mgr3p, most Mgr1p-myc was in a very large complex with both Mgr3p and Yme1p (Figure 4B, lane 1, white arrow). In contrast, when mitochondria from cells lacking Yme1p were used, Mgr1p-myc migrated at ~450 kDa (Figure 4B, lane 2, black arrow) (Dunn *et al.*, 2006). However, the ~450-kDa band containing Mgr1p-myc disappeared when mitochondria were isolated from *yme1Δ mgr3Δ* (Figure 4B, lane 3) cells, indicating that the ~450-kDa subcomplex contains both Mgr1p and Mgr3p. Mgr3p-Mgr1p subcomplex assembly is also demonstrated by experiments tracking the Mgr3p protein. In mitochondria from cells expressing Mgr1p and Yme1p (Supplemental Figure S3, lane 1), Mgr3p-His6 migrated similarly to Mgr1p and Yme1p in a band above the 667-kDa marker. However, in the absence of Yme1p (Supplemental Figure S3, lane 2), Mgr3p-His6 was found in the ~450-kDa form. In mitochondria lacking Mgr1p, Mgr3p-His6 was found in an even smaller, ~200-kDa band (Supplemental Figure S3, lane 3) that is unaffected by further deletion of Yme1p (Supplemental Figure S3, lane 4). We conclude that Mgr3p and Mgr1p normally interact with i-AAA, but Mgr1p and Mgr3p remain stably bound in the absence of Yme1p.

Mgr1p Tethers the Mgr3p-Mgr1p Subcomplex to the i-AAA Protease

Although Mgr1p can bind to Yme1p in mitochondria lacking Mgr3p, the Mgr3p-Yme1p interaction is Mgr1p-dependent. When Mgr1p-myc was precipitated from digitonin-treated

Figure 3. Mgr3p is part of the i-AAA machinery. (A) Yme1p and Mgr1p coprecipitate with Mgr3p. Mitochondria isolated from cells expressing both Mgr1p-myc and Mgr3p-His6 (*MGR3-HIS6*; strain RJ2161) were solubilized, and the Mgr3p-His6 protein precipitated with Ni-NTA agarose. Ten percent of the starting lysate (L) and 100% of the pellet (P) were immunoblotted using antibodies to the His6 tag (Mgr3p-His6), the myc tag (Mgr1p-myc), or to Yme1p. As a control, mitochondria from cells expressing Mgr1p-myc, but lacking Mgr3p-His6 (*MGR3*; RJ1970), were used. (B) Mgr1p, Mgr3p, and Yme1p comigrate during BN-PAGE. Mitochondria were isolated from RJ2161, expressing Mgr3p-His6 and Mgr1p-myc. After solubilization in digitonin buffer, protein complexes were separated by BN-PAGE on a 3–13% gradient gel, and separate lanes from the same gel were immunoblotted with antiserum to Yme1p, the myc epitope of Mgr1p-myc, or the His6 epitope of Mgr3p-His6. (C) Loss of Mgr3p alters the distribution of Yme1p in the i-AAA complexes. Mitochondria were isolated from WT (RJ1962), *mgr1Δ* (RJ1961), *mgr3Δ* (RJ2150), and *mgr1Δ mgr3Δ* (RJ2152) cells and solubilized. Protein complexes were separated on a 3 to 10.5% gradient blue native gel and analyzed with antibodies to Yme1p. Molecular weight standards run in an adjacent lane are indicated. The white and black arrows denote the larger and smaller forms of the i-AAA complex, respectively. Asterisk (*) marks protein not resolvable by BN-PAGE.

mitochondria, the amount of Yme1p coprecipitated was reduced by an average of 64% ($n = 5$) in mitochondria lacking Mgr3p (Figure 5A, lane 4) in comparison with mitochondria containing Mgr3p (Figure 5A, lane 2). This Mgr1p–Yme1p interaction in the absence of Mgr3p was not apparent using BN-PAGE analysis (Figure 4B, lane 4); perhaps the weaker interaction between Mgr1p and Yme1p in the absence of Mgr3p cannot survive BN-PAGE conditions.

Although some interaction between Mgr1p and Yme1p, albeit reduced, can be detected in the absence of Mgr3p, we discovered that coprecipitation of Mgr3p and Yme1p absolutely required the Mgr1 protein. Yme1p was found in the pellet fraction along with Mgr3p-His6 only when mitochondria contained Mgr1p (Figure 5B, lane 2) and not when mitochondria were isolated from *mgr1Δ* cells (Figure 5B, lane 4). Thus, our results suggest that Mgr3p and Mgr1p interact with the Yme1p-containing i-AAA complex via the Mgr1 protein.

Protein Degradation Is Defective in Mitochondria Lacking Mgr3p

Like *mgr1Δ* mutants (Dunn *et al.*, 2006), mitochondria lacking Mgr3p are defective in the turnover of two known

i-AAA substrates. First, we examined the proteolysis of Yta10(161)-DHFR^{MUT}, a fusion between the amino-terminal region of the IM protein Yta10p and a loosely folded form of dihydrofolate reductase (DHFR^{MUT}). After import into isolated mitochondria, Yta10(161)-DHFR^{MUT} is rapidly degraded by the i-AAA protease (Leonhard *et al.*, 1999). We incubated Yta10(161)-DHFR^{MUT} with mitochondria isolated from wild-type, *mgr1Δ*, *mgr3Δ*, and *yme1Δ* cells, and we then monitored Yta10(161)-DHFR^{MUT} levels over time. After import into mitochondria, Yta10(161)-DHFR^{MUT} is processed by the matrix-localized processing protease and occurs as a lower molecular weight doublet (Dunn *et al.*, 2006). Because the significance of these two forms is not known, we quantified both bands together in our analyses (Figure 6A). In wild-type mitochondria, Yta10(161)-DHFR^{MUT} is quickly degraded, with a half-life of ~15 min. In contrast, in mitochondria from the *mgr3Δ*, *mgr1Δ* or *yme1Δ* mutant, proteolysis is greatly inhibited. Little or no turnover of Yta10(161)-DHFR^{MUT} was seen in mitochondria lacking Mgr3p, Mgr1p or Yme1p during the 60-min incubation.

We also found that Mgr3p is required for rapid degradation of a Nde1p-HA fusion protein in intact yeast cells. When the hemagglutinin (HA) epitope is fused to Nde1p, a

Figure 4. Mgr3p and Mgr1p form a subcomplex in the absence of Yme1p. (A) Mgr3p and Mgr1p coprecipitate in mitochondria lacking Yme1p. Mitochondria isolated from *YME1* (RJ2161) and *yme1Δ* (RJ2183) cells, which also express Mgr3p-His6 and Mgr1p-myc, were solubilized and Mgr3p-His6 precipitated using Ni-NTA beads. Ten percent of the starting lysate (L) and 100% of the pellet (P) were immunoblotted using antibodies to the His6 tag (Mgr3p-His6), the myc tag (Mgr1p-myc), or to Yme1p. (B) Mgr1p migrates at ~450-kDa without Yme1p. Mitochondria were isolated from a series of strains expressing Mgr1p-myc: otherwise wild-type strain RJ1970, *yme1Δ* strain RJ1972, *yme1Δ mgr3Δ* strain RJ2214, and *mgr3Δ* strain RJ2148. After solubilization, protein complexes were separated by BN-PAGE on a 3–13% gradient gel and immunoblotted with antiserum recognizing Mgr1p-myc. The white arrow marks the Mgr1p-containing complex when Yme1p and Mgr3p are present, and the black arrow marks the ~450-kDa complex in Yme1p's absence.

mitochondrial NADH dehydrogenase in the IM, the resulting Nde1-HA protein is unstable and rapidly degraded by the i-AAA complex (Augustin *et al.*, 2005). We engineered the Nde1p-HA construct into wild-type, *mgr1Δ*, *mgr3Δ*, *mgr1Δmgr3Δ*, and *yme1Δ* cells. We then added cycloheximide to cultures of the five strains to stop protein synthesis and monitored Nde1p-HA levels at different time points

Figure 5. Mgr1p links the Mgr3p-Mgr1p subcomplex to Yme1p. (A) Mgr1p and Yme1p coprecipitate from mitochondria lacking Mgr3p. Mitochondria from *MGR3* (RJ1970) and *mgr3Δ* (RJ2148) cells expressing Mgr1p-myc were solubilized, and Mgr1p-myc was precipitated using beads coupled to anti-myc antibodies. Ten percent of the starting lysate (L) and 100% of the pellet (P) were immunoblotted using antibodies to the myc tag (Mgr1p-myc) or to Yme1p. (B) Mgr3p and Yme1p do not interact in the absence of Mgr1p. Mitochondria from *MGR1* (RJ2175) and *mgr1Δ* (RJ2186) cells expressing Mgr3p-His6 were solubilized, and Mgr3p-His6 precipitated with Ni-NTA beads. Ten percent of L and 100% of P were immunoblotted using antibodies to the myc tag (Mgr3p-His6) or to Yme1p.

after translation arrest (Figure 6B). In wild-type cells, Nde1p-HA was rapidly degraded with a half-life of ~25 min. As described previously (Augustin *et al.*, 2005; Dunn *et al.*, 2006), Nde1p-HA was completely stabilized in *yme1Δ* cells, and cells lacking Mgr1p were inhibited in turnover but not as severely as *yme1Δ* cells. Loss of Mgr3p also significantly reduced the rate at which the i-AAA complex degraded Nde1p-HA; Nde1p-HA exhibited a half-life of ~36 min in the *mgr3Δ* mutant, compared with ~25 min in wild-type cells. Our studies with intact cells and isolated mitochondria indicate that Mgr3p, like Mgr1p, plays a critical, but not essential, role in the proteolysis of i-AAA substrates. We also found that there was no additive effect of combining *mgr1Δ* and *mgr3Δ* deletions (Figure 6B). These data are in agreement with the more severe phenotypes seen in *yme1Δ* strains compared with *mgr3Δ*, *mgr1Δ*, and *mgr1Δmgr3Δ* mutants. In particular, although *yme1Δ* is temperature-sensitive on nonfermentable medium and cold-sensitive on rich glucose medium, the *mgr1Δ* or *mgr3Δ* single mutants and the *mgr1Δmgr3Δ* double mutant are not cold-sensitive or temperature sensitive (Supplemental Figure S4).

In preliminary studies, we found that the amount of full-length Cox2p, a substrate of the i-AAA complex (Nakai *et al.*, 1995), is normal in *mgr1Δ*, *mgr3Δ*, and *yme1Δ* mutants under conditions where the all members of the cytochrome oxidase complex are present. However, we discovered an accumulation of Cox2p breakdown products in these mutants compared with wild type (Dunn, unpublished data), suggesting

Figure 6. Mgr3p is required for full activity of the i-AAA protease. (A) Degradation of Yta10(161)-DHFR^{MUT} is inhibited in *mgr3*Δ mitochondria. ³⁵S-labeled Yta10(161)-DHFR^{MUT} was imported into mitochondria isolated from WT (RJ1962; diamonds), *mgr1*Δ (RJ1961; squares), *mgr3*Δ (RJ2150; triangles), and *yme1*Δ (RJ1974; circles) and incubated for the indicated times. Mitochondria from each time point were subjected to SDS-PAGE and analyzed by phosphorimaging. The two processed forms of Yta10(161)-DHFR^{MUT} (asterisk) were quantified and plotted. Values shown are the average of three independent experiments. Error bars represent SD. (B) Cells lacking Mgr3p are defective in Nde1p-HA turnover. WT (RJ2077; diamonds), *mgr1*Δ (RJ2078; squares), *mgr3*Δ (RJ2153; triangles), *mgr1*Δ*mgr3*Δ (RJ2163; circles), and *yme1*Δ (RJ2079; asterisks) strains, each expressing Nde1p-HA, were grown to mid-log phase, and then protein synthesis was inhibited with cycloheximide. At the indicated times, cells were lysed, and Nde1p-HA was assayed by immunoblotting with antibodies to the HA-epitope. Values shown are the average of three independent experiments. Error bars represent SD.

a possible role for Mgr1p and Mgr3p in the degradation pathway of Cox2p.

Maximal Association of an Unfolded Substrate with Yme1p Requires Mgr3p and Mgr1p

To further probe the role of Mgr3p and Mgr1p, we examined the association of the folding-defective Yta10(161)-DHFR^{MUT} protein to Yme1p in *mgr1*Δ and *mgr3*Δ mutants. For these studies, we imported ³⁵S-labeled Yta10(161)-DHFR^{MUT} into mitochondria in the presence of ATP.

Then, to inhibit Yme1p-dependent degradation, mitochondria were washed and resuspended in buffer lacking ATP. After further incubation, mitochondria were solubilized in digitonin-containing buffer and binding of Yta10(161)-DHFR^{MUT} to a fully functional Yme1p-His6 fusion protein was monitored using coprecipitation analyses. Consistent with a previous report (Leonhard *et al.*, 1999), we found that in mitochondria containing both Mgr1p and Mgr3p, the imported Yta10(161)-DHFR^{MUT} protein pelleted along with Yme1p-His6 (Figure 7). Al-

Figure 7. Yme1p binding to substrate is reduced in mitochondria lacking Mgr1p or Mgr3p. ^{35}S -labeled Yta10(161)-DHFR^{MUT} was imported into mitochondria isolated from otherwise wild-type strain RJ2195, *mgr1Δ* strain RJ2199, or *mgr3Δ* strain RJ2200, each expressing Yme1p-His6. Mitochondria from strain RJ1962, which lacks Yme1p-His6, were used as a control. After incubation, mitochondria were solubilized and Yme1p-His6 precipitated using Ni-NTA agarose. Ten percent of the starting lysate (L) and 100% of the pellet (P) were subjected to SDS-PAGE, and the ^{35}S -labeled, processed substrate [m-Yta10(161)-DHFR^{MUT}] that coprecipitated with Yme1p-His6 was detected by phosphorimaging. After subtraction of nonspecific precipitation from control mitochondria, the amount of m-Yta10(161)-DHFR^{MUT} in the pellet from YME1-HIS6 mitochondria was set to 1, and error bars represent the SD of five independent experiments.

though the amount of Yta10(161)-DHFR^{MUT} that associated with Yme1p-His6 only averaged ~4% of the total amount imported, this interaction is significant. In particular, when mitochondria lacked the Yme1p-His6 fusion protein, Yta10(161)-DHFR^{MUT} did not pellet with the Ni-NTA beads. Moreover, when we precipitated Atp21p-His6, an abundant IM protein carrying the hexahistidine tag, only background levels of Yta10(161)-DHFR^{MUT} were found in the pellet (Supplemental Figure S5A).

Binding of Yta10(161)-DHFR^{MUT} to Yme1p was reduced when mitochondria lacked either Mgr3p or Mgr1p. When mitochondria isolated from *mgr1Δ* or *mgr3Δ* mutants were used, <1% of the imported Yta10(161)-DHFR^{MUT} protein coprecipitated with Yme1p-His6 (Figure 7). Although binding of Yta10(161)-DHFR^{MUT} to Yme1p-His6 was lower without Mgr1p or Mgr3p, the amount of Yta10(161)-DHFR^{MUT} in the pellet was clearly greater than background levels. Thus, we conclude that Mgr1p and Mgr3p play an important, but not absolute, role in substrate-Yme1p interaction.

Mgr1p and Mgr3p Bind Substrate in the Absence of Yme1p

We then investigated substrate binding to the Mgr1 and Mgr3 proteins. Using antibodies against the myc epitope, we found that ~6% of imported Yta10(161)-DHFR^{MUT} coprecipitated with Mgr1p-myc from solubilized mitochondria (Figure 8A). This binding is specific, because Yta10(161)-DHFR^{MUT} was not found in the pellet fraction when mitochondria lacked myc-tagged Mgr1p (Figure 8A) or when an abundant Atp21p-myc fusion protein was precipitated from mitochondrial extracts (Supplemental Figure S5B). Surprisingly, similar amounts of Yta10(161)-DHFR^{MUT} coprecipitated with Mgr1p-myc in mitochondria lacking Yme1p, Mgr3p, or both Yme1p and Mgr3p (Figure 8A). Similar to our results with Mgr1p, Mgr3p can bind substrate in the absence of both Yme1p and Mgr1p. Approximately 4% of imported substrate coprecipitated with Mgr3p-His6. Comparable amounts of Yta10(161)-DHFR^{MUT} coprecipitated with Mgr3p-His6 in mitochondria expressing Yme1p and Mgr1p or in mitochondria lacking Yme1p, Mgr1p, or both Yme1p and Mgr1p (Figure 8B). Our results suggest that both Mgr1p and Mgr3p are required for the maximal binding of at least one substrate to Yme1p. Furthermore, because both Mgr1p and Mgr3p associate with Yta10(161)-DHFR^{MUT} in the absence of Yme1p, our studies raise the possibility that Mgr1p and Mgr3p function in targeting substrates to the i-AAA protease.

DISCUSSION

By screening a set of yeast knockouts encoding uncharacterized mitochondrial proteins (Sickmann *et al.*, 2003; Zahedi *et al.*, 2006), we uncovered *MGR3*, a new gene required for cell growth without mtDNA. We found that the Mgr3 protein associates with both Mgr1p and Yme1p in the mitochondrial IM, and we discovered that mitochondria lacking Mgr3p were defective in Yme1p-mediated protein turnover, as demonstrated previously for the *mgr1Δ* mutant (Dunn *et al.*, 2006). We conclude that Mgr3p is required for full activity of the i-AAA protease. Because *MGR3* was missed in our earlier screen for petite-negative yeast mutants (Dunn *et al.*, 2006), our prior screen clearly was not exhaustive and additional petite-negative mutants may await discovery. Interestingly, findings from a genome-wide chemical screen (Hillenmeyer *et al.*, 2008) also support a common function for Mgr1p and Mgr3p. In this study, *mgr1Δ* and *mgr3Δ* mutants behaved more similarly to one another than to nearly 5000 other yeast knockout strains in their response to hundreds of different drug treatments. We note that *mgr3Δ* was recently reported to have a weak synthetic fitness interaction with *gem1Δ* (Frederick *et al.*, 2008), which lacks a protein required for wild-type mitochondrial shape and inheritance (Frederick *et al.*, 2004). This synthetic growth defect in *gem1Δ mgr3Δ* double mutants, however, may be due to the combination of the increased rate of mtDNA loss in *gem1Δ* mutants and the petite-negative phenotype of *mgr3Δ*. In support of this view, three of the five strongest synthetic fitness interactions with *gem1Δ* are with mutations that also cause a petite-negative phenotype: *fmp13Δ* (Supplemental Table S1), *tom70Δ* (Dunn and Jensen, 2003), and *phb2Δ* (Dunn *et al.*, 2006).

Our blue native gels results suggest that there are at least two different complexes containing the i-AAA subunit Yme1p. The largest resolvable form, which we call the "i-AAA supercomplex," contains Yme1p, Mgr1p, and Mgr3p, whereas the smaller, i-AAA core complex lacks Mgr1p and Mgr3p. Both complexes migrated well above our

Figure 8. Mgr1p and Mgr3p bind substrate in the absence of Yme1p. (A) Mgr1p binding. ^{35}S -labeled Yta10(161)-DHFR^{MUT} was imported into mitochondria isolated from the following strains expressing Mgr1p-myc: otherwise, wild-type strain RJ1970, *mgr3Δ* strain RJ2148, *yme1Δ* strain RJ1972, or *yme1Δ mgr3Δ* strain RJ2214. After incubation, mitochondria were solubilized, and Mgr1p-myc was precipitated. Ten percent of the mitochondrial lysate (L) and 100% of the pellet fraction (P) were subjected to SDS-PAGE, and the coprecipitated, processed m-Yta10(161)-DHFR^{MUT}, and visualized by phosphorimaging. The amount of substrate in the pellet fraction from MGR1-MYC mitochondria was set to 1 after subtraction of nonspecific substrate precipitation from mitochondria isolated from control strain RJ1962, and error bars represent the SD of three independent experiments. (B) Mgr3p binding. Yta10(161)-DHFR^{MUT} was imported into mitochondria isolated from the following strains expressing Mgr3p-His6: otherwise, wild-type strain RJ2161, *yme1Δ* strain RJ2183, and *yme1Δ mgr1Δ* strain RJ2217. Strains expressing Mgr1p contained the myc-tagged variant. The relative amount of m-Yta10(161)-DHFR^{MUT} precipitated by Mgr3p-His6 was graphed, with the amount in the pellet fraction from MGR3-H6 mitochondria set to 1 after subtraction of nonspecific precipitation from RJ1962 control mitochondria. Error bars represent the SD of three independent experiments.

largest molecular mass standard of 667 kDa, preventing us from accurately determining their sizes. However, because previous gel filtration analyses found only a single ~850-kDa complex (Leonhard *et al.*, 1996) consisting solely of Yme1p subunits (Langer, personal communication), we presume that the higher mobility Yme1p band found in mitochondria lacking Mgr3p or Mgr1p after BN-PAGE analysis represents this ~850-kDa form. Structural studies indicate that members of the AAA family frequently form hexameric ATPase ring structures (Baker and Sauer, 2006). If this is true for the i-AAA protease, then it is likely that the ~850-kDa complex is composed of at least one hexamer, composed of 82-kDa Yme1p subunits. However, because these studies are done in the presence of detergent, it is difficult to determine precise complex sizes. Discerning the organization of the i-AAA supercomplex is even more problematic. In the absence of the i-AAA core complex, Mgr3p and Mgr1p form a subcomplex of ~450-kDa. Therefore, it is likely that the largest resolvable species on our blue native gels represents a connection between the 850-kDa Yme1p multimer and the 450-kDa Mgr3p-Mgr1p-containing subcomplex. Because the Mgr3p-Yme1p interaction requires the Mgr1 protein, we speculate that the Mgr3p-Mgr1p subcomplex is tethered to the Yme1p-containing complex via the Mgr1 subunit. Although our pull-down studies confirm Mgr3p-Mgr1p-

Yme1p interactions, they also suggest that the interplay between the three proteins may be dynamic. For example, only ~10% of Mgr3p, Mgr1p, and Yme1p coprecipitate with each other. Finally, our immunoblots commonly show that some amount of Mgr1p, Mgr3p, and Yme1p is incapable of entering the blue native separating gel, suggesting that even larger, so far irresolvable, i-AAA supercomplexes exist. The exact composition and arrangement of i-AAA clearly awaits further study.

Two observations suggest that Mgr3p and Mgr1p play a direct role in the binding of substrates to the i-AAA protease. First, efficient binding of the loosely folded Yta10(161)-DHFR^{MUT} protein to Yme1p requires both Mgr3p and Mgr1p. In the absence of either protein, approximately five-fold less Yta10(161)-DHFR^{MUT} coprecipitates with Yme1p. Second, Mgr3p and Mgr1p can interact with substrate independently of other known subunits. A similar amount of Yta10(161)-DHFR^{MUT} pellets with Mgr1p-myc from mitochondrial extracts containing Yme1p and Mgr3p as from lysates from mitochondria lacking one or both proteins. Likewise, Yme1p and Mgr1p are not necessary for the association of Yta10(161)-DHFR^{MUT} with Mgr3p-His6. Because Mgr3p and Mgr1p interact with substrate independently, it will be interesting to determine if the two proteins function in a concerted or sequential manner. Surprisingly, equilibra-

lent levels of substrate can be coprecipitated with Mgr1p in the presence or in the absence of both Yme1p and Mgr3p; yet, in mitochondria lacking Mgr3p, substrate coprecipitation with Yme1p is substantially reduced despite a detectable Mgr1p–Yme1p interaction in coprecipitation assays. We therefore believe that the Mgr1 protein associated with Yme1p in the absence of Mgr3p is likely to be in an abnormal, perhaps misfolded conformation that leaves it unable to bind substrate and that the substrate/Mgr1p interaction seen in *mgr3Δ* mitochondria is due to substrate interaction with a pool of binding-competent, non-Yme1p-bound Mgr1p.

We note that the phenotypes of cells lacking Mgr1p, Mgr3p, or both proteins are much less severe than the *yme1Δ* mutant. For example, although the *yme1Δ* mutant has a temperature-sensitive growth defect on nonfermentable medium and is cold sensitive on glucose-containing medium, the *mgr1Δ*, *mgr3Δ*, and *mgr1Δmgr3Δ* cells show no growth defects. Moreover, *mgr1Δ* and *mgr3Δ* show more variation in their phenotypes in different strain backgrounds than does the *yme1Δ* knockout. These data are consistent with at least two scenarios: the Mgr1p–Mgr3p subcomplex could play a minor role in the degradation of all endogenous i-AAA substrates, or the Mgr1p–Mgr3p subcomplex could play a critical role in the degradation of a subset of i-AAA targets. The analyses of different endogenous substrates for their dependence upon the Mgr1p–Mgr3p subcomplex is needed to distinguish between these (and other) possibilities.

Our results suggest that Mgr3p and Mgr1p function like some bacterial adaptor proteins, such as SspB, ClpS, and others that recruit substrates to specific AAA proteases (Baker and Sauer, 2006). The SspB adaptor promotes efficient proteolysis by binding to both the exposed ssrA-tag of target proteins and to the ClpXP protease, stimulating degradation at low concentrations of substrate (Levchenko *et al.*, 2000, 2003, 2005; Wah *et al.*, 2002). Like Mgr3p and Mgr1p, SspB is not essential for ClpXP proteolysis, but SspB increases turnover rates by severalfold (Levchenko *et al.*, 2000; Flynn *et al.*, 2004). Likewise, the ClpS adaptor enhances the degradation of N-end rule substrates by the ClpAP protease at low substrate concentrations (Erbse *et al.*, 2006; Wang *et al.*, 2007). However, ClpS also inhibits degradation of ssrA-tagged proteins (Dougan *et al.*, 2002). It is unclear whether Mgr1p or Mgr3p could, like ClpS, promote the degradation of some substrates while preventing the degradation of others. Finally, we note the additional possibility that Mgr3p and Mgr1p promote Yme1p nucleotide hydrolysis upon binding to substrate, similar to yeast Vta1p, which interacts with the Vps4p AAA protein at the endosomal membrane to promote its assembly and stimulate its ATPase activity (Azmi *et al.*, 2006; Lottridge *et al.*, 2006).

Recent evidence suggests that two distinct regions of the Yme1 protein are capable of binding to substrates. It was previously shown that an N-terminal, juxtamembrane portion of Yme1p lacking the AAA and protease domains, Yme1(1-313)p, is capable of binding to unfolded substrate at the mitochondrial inner membrane (Leonhard *et al.*, 2000). Additionally, recent *in vivo* and *in vitro* work demonstrated the ability of a helical region at the very C terminus of Yme1p, Yme1(650-709)p, to bind substrate in the absence of both the AAA and protease domain (Graef *et al.*, 2007). Interestingly, our preliminary data (Dunn, unpublished data) suggest that Mgr1p is able to bind to the Yme1(1-313) truncation, raising the possibility that Mgr1p or Mgr3p might directly mediate substrate binding to this surface. Future experiments should address how Mgr3p or Mgr1p contribute or control substrate binding to these two separate domains of Yme1p.

Although we have uncovered new components contributing to i-AAA activity, it remains puzzling why protein turnover at the IM is required for mtDNA-dependent yeast cell growth. In cells lacking mtDNA, the electron transport chain and the F_0 portion of the ATP synthase are nonfunctional, and the F_1 portion of the ATP synthase becomes critical for generating the IM potential (Chen and Clark-Walker, 2000). In this case, $\Delta\Psi$ now results from the electrogenic exchange of cytosolic ATP⁻⁴ for ADP⁻³ produced by the matrix-localized F_1 -ATPase (Giraud and Velours, 1997; Chen and Clark-Walker, 2000). In petite mutants, i-AAA activity is required for efficient F_1 -ATPase function. Mitochondria lacking Yme1p have reduced ATPase activity and are deficient in the ATP-driven generation of $\Delta\Psi$ (Kominsky *et al.*, 2002), whereas constructs that express forms of the F_1 -ATPase with increased activity restore mtDNA independence to the *yme1Δ* mutant (Kominsky *et al.*, 2002; Dunn *et al.*, 2006). It has been suggested that the i-AAA protease degrades one or more inhibitors of the F_1 -ATPase. However, this scenario is complicated by the fact that the catalytic domains of i-AAA and the F_1 -ATPase are on opposite sides of the IM. Alternatively, some evidence suggests that peptides released from the mitochondria as a result of proteolysis could act in mitochondria-to-nucleus communication (Arnold *et al.*, 2006; Haynes *et al.*, 2007). Perhaps a putative signaling pathway, activated by i-AAA proteolysis products, plays a critical role in petite cells. The identification of additional substrates for the i-AAA protease complex will help help unravel the connection between mitochondrial protein quality control and mtDNA-independent cell growth.

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REFERENCES

- Adams, A., Gottschling, D., Kaiser, C., and Stearns, T. (1997). *Methods in yeast genetics*. Plainview, NY: Cold Spring Harbor Laboratory Press.
- Arnold, I., Wagner-Ecker, M., Ansorge, W., and Langer, T. (2006). Evidence for a novel mitochondria-to-nucleus signalling pathway in respiring cells lacking i-AAA protease and the ABC-transporter Mdl1. *Gene* 367, 74–88.
- Attardi, G., and Schatz, G. (1988). Biogenesis of mitochondria. *Annu. Rev. Cell Biol.* 4, 289–333.
- Augustin, S., Nolden, M., Muller, S., Hardt, O., Arnold, I., and Langer, T. (2005). Characterization of peptides released from mitochondria: evidence for constant proteolysis and peptide efflux. *J. Biol. Chem.* 280, 2691–2699.
- Azmi, I., Davies, B., Dimaano, C., Payne, J., Eckert, D., Babst, M., and Katzmann, D. J. (2006). Recycling of ESCRTs by the AAA-ATPase Vps4 is regulated by a conserved VSL region in Vta1. *J. Cell Biol.* 172, 705–717.
- Baker, T. A., and Sauer, R. T. (2006). ATP-dependent proteases of bacteria: recognition logic and operating principles. *Trends Biochem. Sci.* 31, 647–653.
- Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14, 115–132.
- Chen, X. J., and Clark-Walker, G. D. (1999). Alpha and beta subunits of F_1 -ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 262, 898–908.
- Chen, X. J., and Clark-Walker, G. D. (2000). The petite mutation in yeasts: 50 years on. *Int. Rev. Cytol.* 194, 197–238.
- Daum, G., Bohni, P. C., and Schatz, G. (1982). Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* 257, 13028–13033.

- Dougan, D. A., Reid, B. G., Horwich, A. L., and Bukau, B. (2002). ClpS, a substrate modulator of the ClpAP machine. *Mol. Cell* 9, 673–683.
- Dunn, C. D., and Jensen, R. E. (2003). Suppression of a defect in mitochondrial protein import identifies cytosolic proteins required for viability of yeast cells lacking mitochondrial DNA. *Genetics* 165, 35–45.
- Dunn, C. D., Lee, M. S., Spencer, F. A., and Jensen, R. E. (2006). A genome-wide screen for petite-negative yeast strains yields a new subunit of the i-AAA protease complex. *Mol. Biol. Cell* 17, 213–226.
- Dupont, C. H., Mazat, J. P., and Guerin, B. (1985). The role of adenine nucleotide translocation in the energization of the inner membrane of mitochondria isolated from rho + and rho degree strains of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* 132, 1116–1123.
- Emtage, J. L., and Jensen, R. E. (1993). MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. *J. Cell Biol.* 122, 1003–1012.
- Erbse, A., Schmidt, R., Bornemann, T., Schneider-Mergener, J., Mogk, A., Zahn, R., Dougan, D. A., and Bukau, B. (2006). ClpS is an essential component of the N-end rule pathway in *Escherichia coli*. *Nature* 439, 753–756.
- Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* 5, 3610–3616.
- Flynn, J. M., Levchenko, I., Sauer, R. T., and Baker, T. A. (2004). Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. *Genes Dev.* 18, 2292–2301.
- Frederick, R. L., McCaffery, J. M., Cunningham, K. W., Okamoto, K., and Shaw, J. M. (2004). Yeast Miro GTPase, Gem1p, regulates mitochondrial morphology via a novel pathway. *J. Cell Biol.* 167, 87–98.
- Frederick, R. L., Okamoto, K., and Shaw, J. M. (2008). Multiple pathways influence mitochondrial inheritance in budding yeast. *Genetics* 178, 825–837.
- Giaever, G. *et al.* (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 418, 387–391.
- Giraud, M. F., and Velours, J. (1997). The absence of the mitochondrial ATP synthase delta subunit promotes a slow growth phenotype of rho- yeast cells by a lack of assembly of the catalytic sector F1. *Eur. J. Biochem.* 245, 813–818.
- Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D. R., and Marmur, J. (1970). The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. *J. Mol. Biol.* 52, 323–335.
- Graef, M., Seewald, G., and Langer, T. (2007). Substrate recognition by AAA+ ATPases: distinct substrate binding modes in ATP-dependent protease Yme1 of the mitochondrial intermembrane space. *Mol. Cell Biol.* 27, 2476–2485.
- Harlow, E., and Lane, D. (1999). Using Antibodies: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Hase, T., Muller, U., Riezman, H., and Schatz, G. (1984). A 70-kD protein of the yeast mitochondrial outer membrane is targeted and anchored via its extreme amino terminus. *EMBO J.* 3, 3157–3164.
- Haynes, C. M., Petrova, K., Benedetti, C., Yang, Y., and Ron, D. (2007). ClpP mediates activation of a mitochondrial unfolded protein response in *C. elegans*. *Dev. Cell* 13, 467–480.
- Hillenmeyer, M. E. *et al.* (2008). The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* 320, 362–365.
- Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* 425, 686–691.
- Jensen, R. E., Dunn, C. D., Youngman, M. J., and Sesaki, H. (2004). Mitochondrial building blocks. *Trends Cell Biol.* 14, 215–218.
- Jensen, R. E., and Yaffe, M. P. (1988). Import of proteins into yeast mitochondria: the nuclear MAS2 gene encodes a component of the processing protease that is homologous to the MAS1-encoded subunit. *EMBO J.* 7, 3863–3871.
- Kerscher, O., Sepuri, N. B., and Jensen, R. E. (2000). Tim18p is a new component of the Tim54p-Tim22p translocase in the mitochondrial inner membrane. *Mol. Biol. Cell* 11, 103–116.
- Koehler, C. M. (2004). New developments in mitochondrial assembly. *Annu. Rev. Cell Dev. Biol.* 20, 309–335.
- Kominsky, D. J., Brownson, M. P., Updike, D. L., and Thorsness, P. E. (2002). Genetic and biochemical basis for viability of yeast lacking mitochondrial genomes. *Genetics* 162, 1595–1604.
- Leonhard, K., Guiard, B., Pellicchia, G., Tzagoloff, A., Neupert, W., and Langer, T. (2000). Membrane protein degradation by AAA proteases in mitochondria: extraction of substrates from either membrane surface. *Mol. Cell* 5, 629–638.
- Leonhard, K., Herrmann, J. M., Stuart, R. A., Mannhaupt, G., Neupert, W., and Langer, T. (1996). AAA proteases with catalytic sites on opposite membrane surfaces comprise a proteolytic system for the ATP-dependent degradation of inner membrane proteins in mitochondria. *EMBO J.* 15, 4218–4229.
- Leonhard, K., Stiegler, A., Neupert, W., and Langer, T. (1999). Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. *Nature* 398, 348–351.
- Levchenko, I., Grant, R. A., Flynn, J. M., Sauer, R. T., and Baker, T. A. (2005). Versatile modes of peptide recognition by the AAA+ adaptor protein SspB. *Nat. Struct. Mol. Biol.* 12, 520–525.
- Levchenko, I., Grant, R. A., Wah, D. A., Sauer, R. T., and Baker, T. A. (2003). Structure of a delivery protein for an AAA+ protease in complex with a peptide degradation tag. *Mol. Cell* 12, 365–372.
- Levchenko, I., Seidel, M., Sauer, R. T., and Baker, T. A. (2000). A specificity-enhancing factor for the ClpXP degradation machine. *Science* 289, 2354–2356.
- Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Lottridge, J. M., Flannery, A. R., Vincelli, J. L., and Stevens, T. H. (2006). Vta1p and Vps46p regulate the membrane association and ATPase activity of Vps4p at the yeast multivesicular body. *Proc. Natl. Acad. Sci. USA* 103, 6202–6207.
- Nakai, T., Yasuhara, T., Fujiki, Y., and Ohashi, A. (1995). Multiple genes, including a member of the AAA family, are essential for degradation of unassembled subunit 2 of cytochrome c oxidase in yeast mitochondria. *Mol. Cell Biol.* 15, 4441–4452.
- Pon, L., Moll, T., Vestweber, D., Marshallsay, B., and Schatz, G. (1989). Protein import into mitochondria: ATP-dependent protein translocation activity in a submitochondrial fraction enriched in membrane contact sites and specific proteins. *J. Cell Biol.* 109, 2603–2616.
- Prokisch, H. *et al.* (2004). Integrative analysis of the mitochondrial proteome in yeast. *PLoS Biol.* 2, e160.
- Rehling, P., Brandner, K., and Pfanner, N. (2004). Mitochondrial import and the twin-pore translocase. *Nat. Rev. Mol. Cell Biol.* 5, 519–530.
- Ryan, K. R., Leung, R. S., and Jensen, R. E. (1998). Characterization of the mitochondrial inner membrane translocase complex: the Tim23p hydrophobic domain interacts with Tim17p but not with other Tim23p molecules. *Mol. Cell Biol.* 18, 178–187.
- Schagger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 199, 223–231.
- Sickmann, A. *et al.* (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl. Acad. Sci. USA* 100, 13207–13212.
- Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 129, 19–27.
- Slonimski, P. P., Perrodin, G., and Croft, J. H. (1968). Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient non-chromosomal "petites". *Biochem. Biophys. Res. Commun.* 30, 232–239.
- Thorsness, P. E., and Fox, T. D. (1993). Nuclear mutations in *Saccharomyces cerevisiae* that affect the escape of DNA from mitochondria to the nucleus. *Genetics* 134, 21–28.
- Thorsness, P. E., White, K. H., and Fox, T. D. (1993). Inactivation of YME1, a member of the ftsH-SEC18-PAS1-CDC48 family of putative ATPase-encoding genes, causes increased escape of DNA from mitochondria in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 13, 5418–5426.
- Tzagoloff, A. (1982). Mitochondria, New York: Plenum Press.
- Wah, D. A., Levchenko, I., Baker, T. A., and Sauer, R. T. (2002). Characterization of a specificity factor for an AAA+ ATPase: assembly of SspB dimers with ssrA-tagged proteins and the ClpX hexamer. *Chem. Biol.* 9, 1237–1245.
- Wang, K. H., Sauer, R. T., and Baker, T. A. (2007). ClpS modulates but is not essential for bacterial N-end rule degradation. *Genes Dev.* 21, 403–408.
- Winston, F., Dollard, C., and Ricupero-Hovasse, S. L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* 11, 53–55.
- Yaffe, M. P., Jensen, R. E., and Guido, E. C. (1989). The major 45-kDa protein of the yeast mitochondrial outer membrane is not essential for cell growth or mitochondrial function. *J. Biol. Chem.* 264, 21091–21096.
- Zahedi, R. P., Sickmann, A., Boehm, A. M., Winkler, C., Zufall, N., Schonfisch, B., Guiard, B., Pfanner, N., and Meisinger, C. (2006). Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. *Mol. Biol. Cell* 17, 1436–1450.