RESEARCH ARTICLE SUMMARY

CELL BIOLOGY

MitoCPR–A surveillance pathway that protects mitochondria in response to protein import stress

Hilla Weidberg* and Angelika Amon*

INTRODUCTION: Mitochondria provide cells with energy and numerous essential metabolites such as lipids, amino acids, iron sulfur clusters, and heme. All mitochondrial functions rely on import of proteins into the organelle because the mitochondrial proteome is almost exclusively encoded by nuclear genes. Given the central importance of mitochondria for cell viability, it is not surprising that cells mount a nuclear response when mitochondrial functions are compromised. These mitochondria-to-nucleus signaling pathways include the mtUPR (mitochondrial unfolded protein response), which triggers expression of mitochondrial chaperones when mitochondrial protein folding is defective, and the UPRam (unfolded protein response activated by mistargeting of proteins) and mPOS (mitochondrial precursor over-accumulation stress) pathways, which reduce translation and induce degradation of unimported proteins in the cytosol when mitochondrial import is impaired. Even though mitochondrial import is central to all mitochondrial functions, no response to protein import defects had been described that protects mitochondria during this stress.

RATIONALE: To determine how cells respond to defects in mitochondrial protein import, we first developed a system in budding yeast with which to specifically inhibit this process. We found that overexpression of proteins that rely on a bipartite signal sequence for their mitochondrial localization inhibited mitochondrial import and led to the accumulation of mitochondrial precursors. Protease protection and carbonate extraction assays that were performed on isolated mitochondria revealed that these unimported proteins accumulated on the mitochondrial surface and in the import channel known as the translocase.

RESULTS: Having developed a system that allowed us to specifically inhibit mitochondrial protein import, we examined the cellular response to this defect. Transcriptome analysis of cells overexpressing bipartite signal–containing proteins identified a gene expression pattern related to the multi-drug resistance response. We termed this response mitochondrial compromised protein import response (mitoCPR). mitoCPR was triggered by protein import defects but not other mitochondrial deficiencies, such as respiratory failure, and was mediated by the transcription factor Pdr3. Our analyses further showed that mitoCPR was critical for the protection of mitochondria during import stress. Cells lacking *PDR3* did not mount a mitoCPR during import stress and accumulated higher levels of unimported proteins on the organelle surface as compared with those of wild-type cells. Consequently, *pdr3*Δ cells exhibited decreased respiratory function and loss of mitochondrial DNA when mitochondrial import was restored. Our results also shed light on the mechanism by which mitoCPR protected mitochondria. Upon mitochondrial import stress, Pdr3 induced expression of Cis1. Coimmunoprecipitation analyses showed that Cis1 recruited the AAA$^+$ adenosine triphosphatase Msp1 to the translocase by binding to the translocase receptor Tom70. There, the two proteins mediated the clearance and proteasomal degradation of proteins that failed to be imported into mitochondria.

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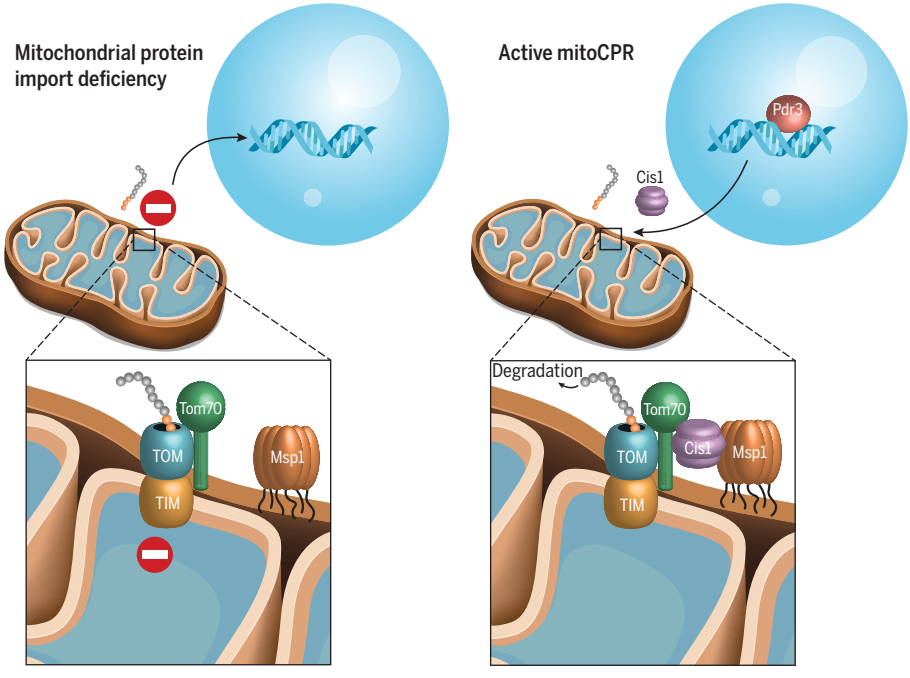
CONCLUSION: We discovered a mitochondrial import surveillance mechanism in budding yeast. This surveillance mechanism, mitoCPR, is activated when mitochondrial import is stalled in order to induce the removal of mitochondrial proteins accumulating on the mitochondrial surface. Clearance of precursors is critical for maintaining mitochondrial functions during import stress. We propose that mitoCPR could be especially important when the import machinery is overwhelmed, as may occur in situations that require the rapid expansion of the mitochondrial compartment. ■



MitoCPR protects mitochondria during import stress. (Left) Mitochondrial protein import deficiency leads to the accumulation of mitochondrial proteins on the organelle's surface and in the translocases. (Right) Pdr3 induces *CIS1* expression. Cis1 binds to the mitochondrial import receptor Tom70 and recruits Msp1 to mediate clearance of unimported precursors from the mitochondrial surface and their proteasomal degradation. This protects mitochondrial functions during import stress.

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RESEARCH ARTICLE

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MitoCPR–A surveillance pathway that protects mitochondria in response to protein import stress

Hilla Weidberg* and Angelika Amon*

Mitochondrial functions are essential for cell viability and rely on protein import into the organelle. Various disease and stress conditions can lead to mitochondrial import defects. We found that inhibition of mitochondrial import in budding yeast activated a surveillance mechanism, mitoCPR, that improved mitochondrial import and protected mitochondria during import stress. mitoCPR induced expression of Cis1, which associated with the mitochondrial translocase to reduce the accumulation of mitochondrial precursor proteins at the mitochondrial translocase. Clearance of precursor proteins depended on the Cis1-interacting AAA$^+$ adenosine triphosphatase Msp1 and the proteasome, suggesting that Cis1 facilitates degradation of unimported proteins. mitoCPR was required for maintaining mitochondrial functions when protein import was compromised, demonstrating the importance of mitoCPR in protecting the mitochondrial compartment.

Mitochondrial function is required for cell viability, producing energy, and many essential biological molecules such as iron-sulfur clusters and heme (*1*). Even though mitochondria contain their own genome, the vast majority of their proteins are encoded by the nucleus. Import of nuclear-encoded proteins into mitochondria is essential for mitochondrial function and cell viability (*1*, *2*). Defects in mitochondrial protein import are associated with various human diseases, such as deafness-dystonia syndrome and Huntington's disease (*3*–*5*). However, even though mitochondrial protein import is essential for all mitochondrial functions, little is known about how cells respond to mitochondrial protein import defects. Recently, two pathways—mPOS (mitochondrial precursor over-accumulation stress) and UPRam (unfolded protein response activated by mistargeting of proteins)—have been identified in yeast that respond to the accumulation of unimported mitochondrial proteins in the cytosol (*6*, *7*). UPRam and mPOS reduce global translation, and UPRam protects the cytosol from proteotoxic effects of unimported proteins by accelerating their degradation. In mammals, the Ubiquilin family of proteins has a similar role in mediating the degradation of mitochondrial transmembrane proteins that fail to get imported and remain in the cytosol (*8*). Whether mechanisms exist that protect mitochondrial functions in the face of mitochondrial import stress is unclear. We identified a response to mitochondrial protein import defects that protected mitochondrial functions by reducing the accumulation of precursor proteins at the mitochondrial surface and translocase. This response was brought about by the transcription factor *PDR3*, which has previously been shown to mediate a multidrug resistance (MDR) response.

The canonical MDR response is conserved from bacteria to mammals (*9*). It protects organisms from xenobiotics and can limit the effectiveness of microbial and cancer chemotherapy (*9*, *10*). In budding yeast, the MDR response is activated by a variety of chemical compounds and is primarily mediated by the two related transcription factors Pdr1 and Pdr3 (*11*–*13*). They induce the expression of several adenosine 5′-triphosphate (ATP)–binding cassette (ABC) transporters to mediate efflux of xenobiotics (*13*). A transcriptional response related to the MDR and specifically mediated by Pdr3 is active in yeast cells with defective mitochondrial DNA (mtDNA) (*14*). In such cells, Pdr3 induces the expression of genes encoding ABC transporters, sphingolipid biosynthesis enzymes, and a number of genes of unknown function (*15*). We show here that Pdr3 mediates a mitochondrial import defect response.

A system to acutely inhibit mitochondrial protein import

All mitochondrial functions depend on proteins being imported from the cytosol into the organelle. Whether pathways exist that monitor import of proteins into mitochondria and elicit a cellular response under conditions of mitochondrial import stress is unknown. To determine whether cells respond to mitochondrial import stress, we examined the consequences of acutely interfering with mitochondrial protein import. Compounds that uncouple the mitochondrial respiratory chain, such as CCCP (carbonyl cyanide *m*-chlorophenyl hydrazine), prevent mitochondrial import, which is dependent on the mitochondrial membrane potential (*2*). However, these same compounds can also affect potential across other cellular membranes and induce a MDR response, which complicates delineating responses specific to mitochondrial import defects. We hypothesized that acute induction of mitochondrial import stress could be achieved without drugs by overloading the mitochondrial import machinery through overexpression of mitochondrial proteins. We overexpressed a number of mitochondrial proteins from the strong galactose-inducible *GAL1-10* promoter and assessed the mitochondrial import of Cox5a, a nuclear-encoded subunit of mitochondrial complex IV. Like most mitochondrial proteins, Cox5a harbors an N-terminal presequence that is cleaved upon import into the mitochondrial matrix (*16*). In untreated cells, mitochondrial import and precursor cleavage was so efficient that the Cox5a preprotein (Cox5a^{pre}) was not detected (Fig. 1A). Upon disruption of membrane potential and hence protein import with CCCP, Cox5a^{pre} accumulated in cells (Fig. 1A).

Overexpression of the majority of mitochondrial proteins did not affect Cox5a processing, but high levels of Psd1, Ccp1, Cyb2, Cox5a, or Tim50 led to Cox5a^{pre} accumulation (Fig. 1A). All five proteins use the same mitochondrial import machinery. They contain a bipartite signal that inhibits translocation into the mitochondrial matrix. This results in the lateral release of proteins out of the inner-membrane translocase TIM23 into the inner membrane itself (*2*). A broad survey of mitochondrial proteins known to contain a bipartite signal confirmed this conclusion (Fig. 1B). By contrast, inner-membrane proteins that use other import mechanisms (for example, the TIM22 pathway) or proteins that translocate across the TIM23 translocase, such as matrix proteins, did not affect Cox5a processing (Fig. 1A). Overexpression of bipartite signal–containing proteins affected import of proteins other than Cox5a. High levels of the bipartite signal–containing protein Psd1 interfered with the processing of a number of presequence-containing proteins whose import is mediated by the TIM23 complex (Fig. 1C). Thus, saturation of the TIM23 lateral diffusion import pathway leads to the accumulation of mitochondrial preproteins.

The accumulation of mitochondrial preproteins could reflect defects in either translocation into mitochondria or presequence cleavage in the matrix. To test the former possibility, we determined the localization of Cox5a^{pre}. Both the mature and the preprotein forms of Cox5a were detected in mitochondrial but not cytosolic fractions after overexpression of *PSD1* or CCCP treatment (Fig. 2A). Addition of proteinase K to the mitochondrial fractions led to loss of Cox5a^{pre} but not mature Cox5a, which resides in the inner membrane with its C terminus facing the intermembrane space. Because Cox5a was detected by using a C-terminal V5 tag in this analysis, we conclude that at least the C terminus of Cox5a^{pre} resides at the surface of mitochondria that faces the cytosol. These results lead to two important

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conclusions. First, overexpressed bipartite signal–containing proteins interfere with mitochondrial protein translocation. Second, the C-terminus of Cox5a^{pre} accumulates at the mitochondrial surface when mitochondrial import is impaired.

Cox5a^{pre} could be peripherally associated with the mitochondrial outer membrane by binding to receptors on the mitochondrial surface, be trapped in the translocase, or be incorrectly inserted into the outer membrane via its transmembrane domain. To determine the exact localization of Cox5a^{pre}, we treated mitochondria preparations with sodium carbonate (pH 11), which extracts peripheral membrane proteins from membranes (*17*). As expected, the inner-membrane localized, mature Cox5a was largely resistant to sodium carbonate extraction, while the peripheral outer membrane protein Cis1 dissociated from mitochondria during this treatment (Fig. 2B). Most of Cox5a^{pre} remained associated with mitochondrial membranes during sodium carbonate treatment, indicating that a large fraction of Cox5a^{pre} was either inappropriately integrated into the outer membrane or stalled in the TOM (translocase of the outer membrane) translocase (Fig. 2B). To distinguish between these possibilities, we investigated the localization of Sod2, a mitochondrial matrix protein that lacks any transmembrane domains. Like Cox5a^{pre}, Sod2^{pre} accumulated at the mitochondrial outer membrane after overexpression of *PSD1*; association of the precursor with mitochondrial fractions was sensitive to proteinase K treatment (fig. S1). Sod2^{pre} was also largely resistant to sodium carbonate extraction (Fig. 2C). By contrast, sodium carbonate treatment solubilized mature matrix-localized Sod2. Thus, during import stress, mitochondrial preproteins are tightly bound to the mitochondrial outer membrane independently of transmembrane domains. This suggests that at least a fraction of the preproteins is stalled in the mitochondrial translocase during import stress.

How do bipartite signal–containing proteins interfere with protein import when overexpressed? To address this question, we determined which bipartite signal element interfered with protein import when overexpressed. Bipartite mitochondrial targeting signals comprise a mitochondrial targeting sequence (MTS) and a hydrophobic segment that directs the protein to the inner membrane. Overexpressed Psd1 lacking its MTS did not inhibit mitochondrial protein import, demonstrating that Psd1 must be imported into mitochondria to interfere with Cox5a import (Fig. 2D). Consistent with this conclusion, Psd1's bipartite signal was sufficient to inhibit Cox5a mitochondrial import. Overexpression of green fluorescent protein (GFP) fused to Psd1's bipartite signal inhibited Cox5a import, whereas a fusion between only Psd1's MTS and GFP did not (Fig. 2D). Thus, when present in excess, bipartite signal–containing proteins interfere with import only when targeted to the inner membrane. This finding indicates that lateral diffusion out of TIM23 translocase is a rate-limiting step in mitochondrial import that can be saturated by overexpressing proteins imported via this route.

Mitochondrial import defects activate the mitoCPR

Does inhibiting protein import elicit a cellular response? To address this question, we examined the transcriptional consequences of overexpressing *PSD1*. Overexpression of *PSD1* up-regulated 217 genes and down-regulated 11 genes by twofold or more (table S1). Among the up-regulated genes was a group of genes previously shown to be induced by the transcription factor Pdr3, but not its close homolog Pdr1, in response to *PSD1* overexpression and loss of mtDNA (*14*, *18*). We identified 19 genes whose induction upon mitochondrial import stress depended on *PDR3* (Fig. 3, A and B, and table S1). This group of genes included MDR response genes such as genes encoding ABC transporters, proteins involved in lipid metabolism and transport, reduced nicotinamide adenine dinucleotide phosphate (NADPH)–dependent enzymes, and a number of proteins of unknown function (Fig. 3A). Other, well-characterized mitochondrial stress responses were, however, not activated by *PSD1* overexpression within the time frame of the experiment. *PSD1*-overexpressing cells did not induce *RTG* (retrograde)–regulated genes, such as *CIT2* and *PDH1*, that are known to be activated in response to defects in Krebs cycle function (table S1) (*19*). The finding that overexpression of *PSD1* inhibited mitochondrial import suggests that it is mitochondrial import defects that elicit this *PDR3*-mediated transcriptional response. The finding that cells lacking mtDNA, which exhibit severe mitochondrial import defects (*20*, *21*), also show that this transcriptional response is consistent with this idea.

To further explore a potential link between the *PDR3*-mediated transcriptional response and mitochondrial import defects, we first asked whether proteins—which, when overexpressed, inhibited mitochondrial import—also induced the *PDR3*-mediated transcriptional response. This was the case. All mitochondrial proteins that caused protein import defects when overexpressed induced the *PDR3*-mediated transcriptional response as determined by up-regulation of the *PDR3*-responsive

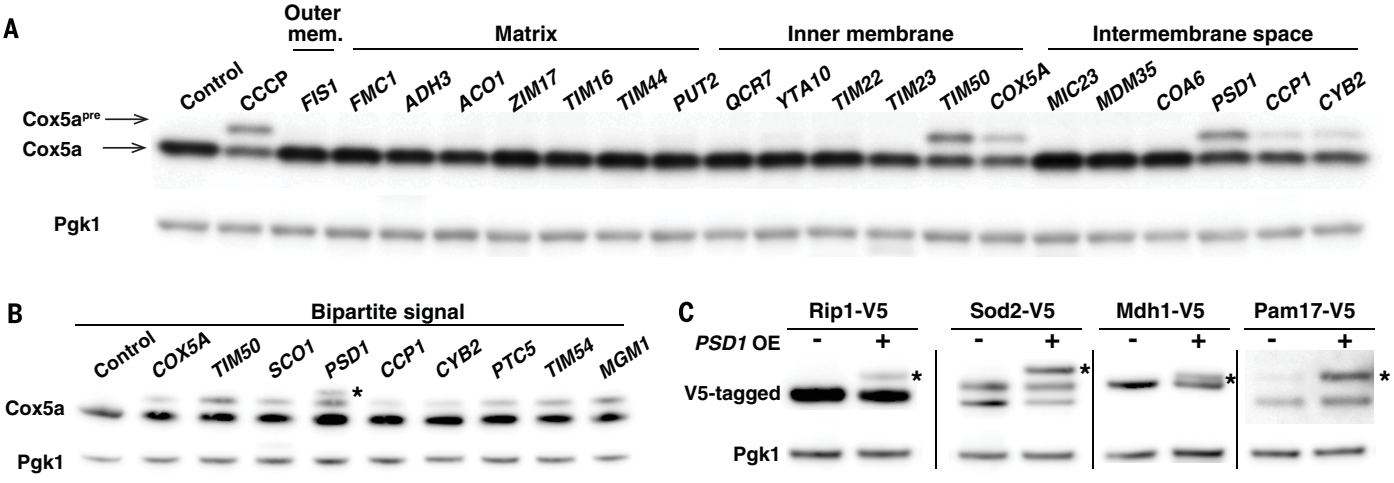

Fig. 1. Overexpression of bipartite signal–containing proteins induces mitochondrial protein import defects. (**A**) Immunoblot of Cox5a-V5 and Cox5a^{pre}-V5 (Cox5a preprotein) in control cells, CCCP-treated cells (20 μM, 1 hour), or cells overexpressing mitochondrial proteins through the addition of galactose for 4 hours. Overexpressed proteins are divided by their localization to the outer membrane (outer mem.), matrix, inner membrane, and intermembrane space compartments. Pgk1 was used as a loading control. (**B**) Same as (A). Asterisk represents a nonspecific band, result of *PSD1* overexpression. (**C**) Immunoblot of Rip1-V5, Sod2-V5, Mdh1-V5, and Pam17-V5 (expressed from their endogenous promoter) in control cells or after overexpression of *PSD1* for 4 hours. Asterisks identify the precursor form of the indicated proteins. OE, overexpression. As previously shown (*50*), Sod2 migrates in SDS–polyacrylamide gel electrophoresis (PAGE) as a doublet under conditions when mitochondria are intact and as a triplet when its cleavage is inhibited.

gene *CIS1*. Conversely, proteins whose overexpression did not interfere with mitochondrial import did not induce *CIS1* (Fig. 3C and fig. S2, A and B). The perfect correlation between the ability to inhibit mitochondrial import and induction of a *PDR3*-mediated transcriptional response was also observed when analyzing cells overexpressing various *PSD1* domains. Cells overexpressing Psd1 that lacked its mitochondrial targeting signal or that harbored an N-terminal V5 tag to prevent targeting of the protein to mitochondria failed to induce *CIS1* (Fig. 3, D and E, and fig. S2, C and D) or any other *PDR3*-mediated transcripts (table S1). By contrast, GFP that was fused to the complete Psd1 bipartite signal induced *CIS1* when overexpressed, whereas GFP that was fused only to Psd1's MTS did not (Fig. 3D and fig. S2C).

The *PDR3*-mediated transcriptional response was not only induced through acute induction of mitochondrial import defects but was also seen in mutants in which mitochondrial import was constitutively impaired. Cells harboring deletions in mtDNA (*rho*$^-$ cells) or lacking mtDNA (*rho0* cells), both of which cause mitochondrial import defects, expressed *CIS1* at an elevated level (Fig. 3F) (*22*). Cells lacking *TAM41*, a gene encoding a cardiolipin biosynthesis enzyme, have severe mitochondrial import defects but intact mtDNA (*23*, *24*). These cells, too, expressed *CIS1* at high levels (Fig. 3F). Not all mitochondrial defects elicited the *PDR3*-mediated transcriptional response. Deletion of genes encoding subunits of respiration complexes III and IV results in respiration defects (*25*, *26*) but did not cause induction of *CIS1* expression (fig. S2E). Our results reveal a tight correlation between mitochondrial import defects and induction of a *PDR3*-mediated transcriptional response.

To further test the hypothesis that induction of the *PDR3*-mediated transcriptional response is caused by mitochondrial import defects, we examined the consequences of suppressing mitochondrial import defects on *CIS1* expression. The *ATP1-111* allele increases membrane potential and improves protein import in *rho0* cells by altering the ATP:ADP (adenosine 5′-diphosphate) ratio between the matrix and the intermembrane space (*20*, *21*, *27*). Introduction of the *ATP1-111* allele into either *rho0*, *rho*$^-$, or *tam41*Δ cells caused a large decrease in *CIS1* expression (Fig. 3G). Thus, either defects in membrane potential or import defects elicit a *PDR3*-mediated transcriptional response. The finding that overexpression of *PSD1* for 4 hours, which is sufficient to induce the *PDR3*-mediated transcriptional response, did not significantly affect mitochondrial membrane potential (Fig. 3B and fig. S2F) suggested that membrane potential defects do not lead to induction of Pdr3 target genes. Thus, mitochondrial import defects cause a *PDR3*-mediated transcriptional response. We termed this response mitoCPR for mitochondrial compromised protein import response.

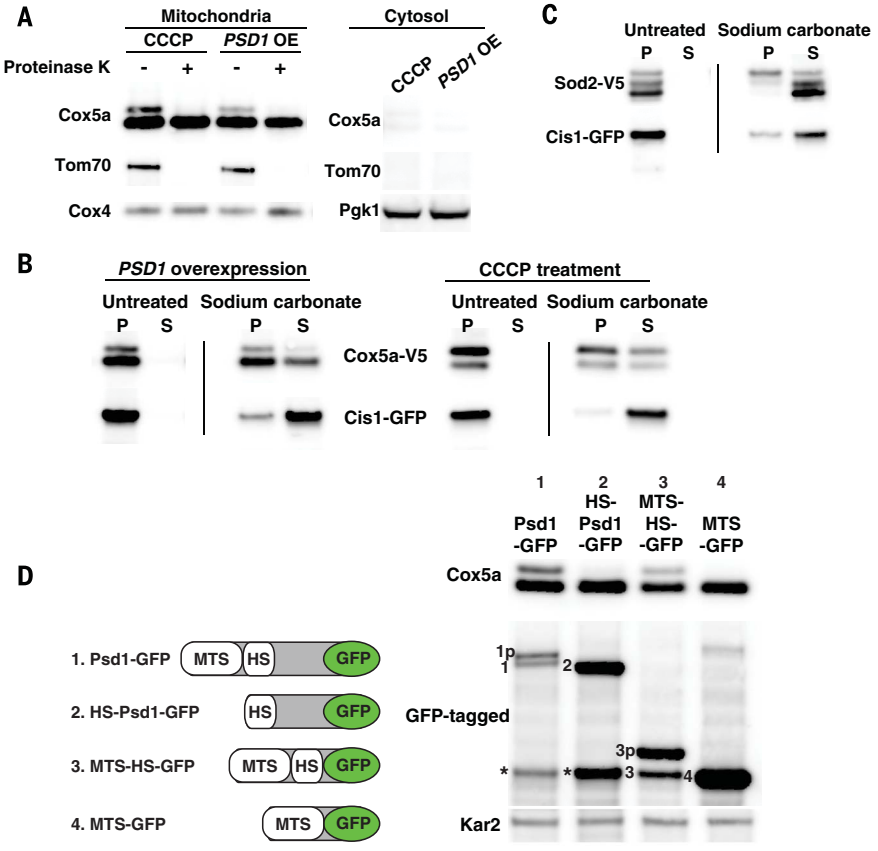

Fig. 2. Mitochondrial precursors accumulate on the surface of the organelle and in the translocase during import stress. (**A**) Mitochondria were isolated by means of differential centrifugation from cells treated with 20 μM CCCP for 1 hour or cells overexpressing *PSD1* for 6 hours. Cox5a-V5, Tom70-mCherry, and Cox4 or Pgk1 were detected in mitochondria and cytosol fractions. Mitochondria were treated with 50 μg/ml of proteinase K. Tom70 served as an outer-membrane control protein; Cox4 served as a matrix control protein. OE, overexpression. (**B**) Mitochondria were isolated from cells expressing *COX5a-V5* and *CIS1-GFP* and overexpressing *PSD1* for 6 hours or cells treated with 20 μM CCCP for 1 hour. Sodium carbonate–treated or –untreated mitochondria were centrifuged so as to separate insoluble proteins [pellet (P)] from soluble proteins [supernatant (S)]. Samples were analyzed by means of immunoblot analysis. Cis1-GFP served as a peripheral outer-membrane protein control. (**C**) Mitochondria were isolated from cells overexpressing *PSD1* for 6 hours. Mitochondria were treated as in (B) in order to analyze Sod2-V5 by means of immunoblot analysis. (**D**) (Left) *PSD1-GFP* constructs used in the analysis. MTS, mitochondrial targeting sequence; HS, hydrophobic segment. (Right) Immunoblot blot of Cox5a-V5 and Psd1-GFP fusion proteins after overexpression of *PSD1-GFP* fusion genes for 4 hours. Kar2 was used as a loading control. Numbering on the immunoblot indicates the mature form of the GFP-tagged proteins. The letter "p" following this number identifies the precursor form of proteins. Asterisks identify a proteolytic cleavage product of Psd1 known as the α subunit (*51*).

The mitoCPR protects mitochondrial functions during import stress.

What is the role of the mitoCPR when mitochondrial protein import is impaired? To address this question, we first determined the consequences of deleting *PDR3* on the fate of Cox5a^{pre} under conditions in which protein import is impaired. As shown above, overexpression of *PSD1* led to the accumulation of Cox5a^{pre} (Fig. 1A). Cox5a^{pre} had a half-life of ~19 min in *PSD1*-overexpressing cells (Fig. 4, A and B). The eventual loss of Cox5a^{pre} in *PSD1*-overexpressing cells could be due to import of the preprotein into mitochondria, cytosolic degradation, or both. Deletion of *PDR3* prolonged the half-life of Cox5a^{pre} (Fig. 4, A and B). Conversely, overexpression of *PDR3* partially suppressed the accumulation of Cox5a^{pre} under conditions of mitochondrial import stress (Fig. 4C). Thus, *PDR3* and by extension mitoCPR are critical for either maintaining some level of mitochondrial import and/or clearing preproteins from the mitochondrial import machinery during import stress.

Next, we determined whether mitoCPR was important for maintaining mitochondrial functions under conditions of import stress. Upon overexpression of *PSD1*, oxygen consumption rate decreased (Fig. 4D and fig. S3A). Deletion of *PDR3* further exaggerated this effect (Fig. 4D and fig. S3A), indicating that *PDR3* is critical for maintaining mitochondrial respiration when mitochondrial import is compromised.

PDR1 and *PDR3* prevent mtDNA loss resulting from mitochondrial fusion defects (*28*). We tested

whether mitoCPR was important for protecting cells from mtDNA loss during import stress. Respiratory competence is a readout of mtDNA integrity. Assaying respiration, however, requires the analysis of colonies. This prerequisite precluded us from inducing mitochondrial import stress through overexpression of *PSD1* because prolonged overexpression of *PSD1* is lethal (fig. S3B). In fact, overexpression of all bipartite signal–containing proteins is lethal (fig. S3C). The mitochondrial Hsp70 chaperone Ssc1 and its cochaperone Mge1 are essential for mitochondrial import (*29*–*31*). We hypothesized that overexpression of *SSC1* or *MGE1* alone would lead to a mitochondrial import defect because the proper ratio of Hsp70 to its cochaperone is crucial for its chaperone activity in bacteria (*32*). Overexpression of *MGE1*, although not lethal (fig. S3B), caused a mild protein import defect comparable with that of cells lacking mtDNA. $Cox5a^{pre}$ did not accumulate in *MGE1*-overexpressing cells or *rho0* cells upon induction of Cox5a expression from the methionine-regulated promoter (*MET25*) (fig. S3D). Nevertheless, mature Cox5a levels were reduced in *GAL-MGE1* and *rho0* cells as compared with control cells, whereas *COX5a* mRNA expression was comparable in all strains (fig. S3, D and E). Thus, less Cox5a is imported into mitochondria in *MGE1*-overexpressing cells, and unimported $Cox5a^{pre}$ is rapidly degraded. Consistent with a mild mitochondrial import defect, overexpression of *MGE1* induced a mitoCPR as determined by elevated *CIS1* levels (as did overexpression of *SSC1*) (fig. S3, F and G).

Having established that overexpression of *MGE1* causes a mild mitochondrial import defect that is not lethal, we examined its effects on mtDNA stability. Overexpression of *MGE1* for 24 hours led to an increase in rho^- cells (Fig. 4E). Inactivation of mitoCPR by deleting *PDR3* caused a threefold increase in cells harboring defective mtDNA (Fig. 4E). Because maintenance of mtDNA largely depends on nuclear-encoded genes (*33*), we conclude that mitochondrial import stress prevented their import. This caused mtDNA damage and the generation of rho^- cells. Furthermore, the mitoCPR protects mtDNA only during import stress. The absence of *PDR3* did not affect respiration or mtDNA maintenance under normal growth conditions. Thus, mitoCPR has a protective role specifically during mitochondrial import stress.

Cis1 protects mitochondria during import stress

One of the most strongly induced genes after mitochondrial import stress is *CIS1* (Fig. 3A) (*34*). *CIS1* overexpression improves cellular fitness in the presence of citrinin, a mycotoxin that reduces mitochondrial membrane potential (*35*). The protein itself, however, neither harbors domains with known functions nor has homologs in higher eukaryotes. Cis1 protein only accumulated under conditions of mitochondrial import stress and was unstable even when expressed (fig. S4, A and B). Cis1 associates with mitochondria in high-throughput localization studies (*36*), which prompted us to investigate whether the protein played a role in protecting mitochondria during import stress. To study Cis1, we placed the gene under the constitutive *TEF2* promoter (fig. S4A). A constitutively expressed Cis1-GFP fusion indeed predominantly localized to the outer membrane of the organelle (Fig. 5, A to C). Cis1 is not predicted to have a transmembrane domain. We conclude that Cis1 associates with the outer mitochondrial membrane facing the cytosol.

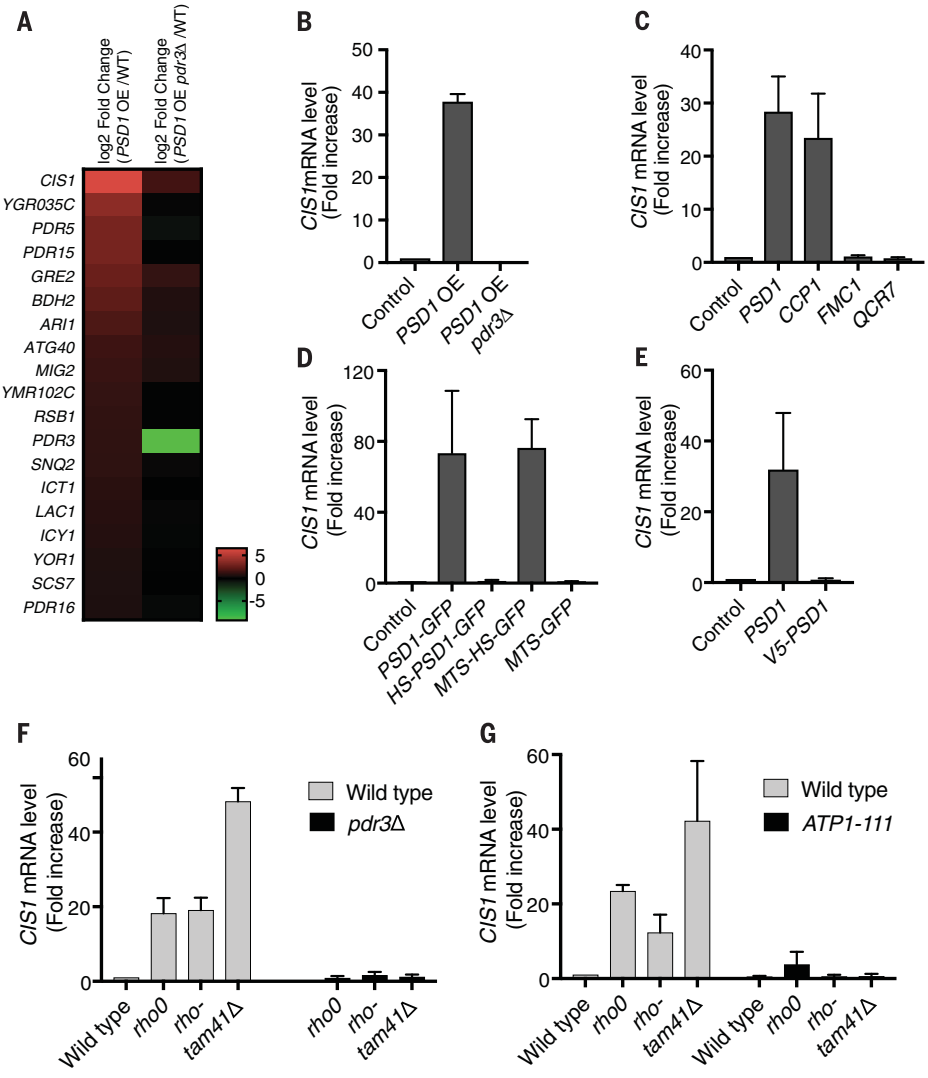

Fig. 3. Inhibition of mitochondrial protein import induces the mitoCPR. (**A**) Gene expression analysis of control wild-type cells and wild-type or *pdr3*Δ cells that overexpressed *PSD1* for 4 hours through galactose induction. The heat map describes the transcription profiles of cells overexpressing *PSD1* and *pdr3*Δ cells overexpressing *PSD1*. The 19 genes shown met the following criteria: (i) genes that exhibited an increase in expression of at least (log2) 0.6 and adjusted *P* values that are equal to or lower than 0.05 in *PSD1*-overexpressed cells versus *PSD1*-overexpressed cells lacking *PDR3*; (ii) genes that exhibited an increase in expression of at least (log2) 0.6 and adjusted *P* values that are equal to or lower than 0.05 in *PSD1*-overexpressed cells versus control cells. WT, wild type. OE, overexpression. (**B**) *CIS1* mRNA levels in wild-type cells, cells overexpressing *PSD1*, or *pdr3*Δ cells overexpressing *PSD1*. *PSD1* expression was induced through the addition of galactose for 4 hours. *n* = 3 experiments; data are mean ± SD. (**C**) *CIS1* mRNA levels in control cells or cells overexpressing mitochondrial proteins through galactose induction (4 hours) were analyzed by means of quantitative reverse transcription polymerase chain reaction (RT-PCR). *n* = 3 experiments; data are mean ± SD. (**D**) Same as (C), after overexpression of *PSD1-GFP* fusion genes for 4 hours. MTS, mitochondrial targeting sequence; HS, hydrophobic segment. *n* = 3 experiments; data are mean ± SD. (**E**) Same as (C), after overexpression of *PSD1* or *V5-PSD1* for 4 hours. *n* = 3 experiments; data are mean ± SD. (**F**) *CIS1* mRNA levels of wild-type, *rho0*, rho^-, and *tam41*Δ cells in the presence or absence of *PDR3*. *n* = 3 experiments; data are mean ± SD. (**G**) Same as (F), in the presence or absence of the *ATP1-111* allele. *n* = 3 experiments; data are mean ± SD.

The expression of Cis1 proved to be important for cells during mitochondrial protein import stress. Although deletion of *CIS1* did not have a noticeable effect on Cox5a^{pre} levels (fig. S4C), it did cause a defect in mtDNA maintenance during mitochondrial import stress caused by *MGE1* overexpression (Fig. 5D). The effects of deleting *CIS1* on the mitoCPR were subtle, presumably because proteins acting in parallel could substitute for *CIS1* function. The expression of *CIS1* from the constitutive *TEF2* promoter, however, had a substantial protective effect during mitochondrial import stress. It led to a decrease in Cox5a^{pre} levels after *PSD1* overexpression and an increase in the levels of mature Cox5a (Fig. 5E).

Drugs such as CCCP could not be used to study the role of *PDR3* during mitochondrial import stress because the drug caused *PDR3*-independent expression of mitoCPR genes, including *CIS1*. *TEF2* is, however, not controlled by any MDR response, which allowed us to explore the role of *CIS1* expressed from the *TEF2* promoter in the mitoCPR using CCCP. We induced expression of *COX5a* from the *MET25* promoter and simultaneously treated cells with CCCP. CCCP treatment partially blocked mitochondrial import, causing Cox5a^{pre} to accumulate. Constitutive expression of *CIS1* prevented this accumulation (Fig. 5, F to H). Constitutive Cis1 had the same effect on the matrix proteins Rmd9, Ilv2, and Mss116 (fig. S4, D and E). Thus, high levels of Cis1 affect precursor levels of many and perhaps all mitochondrial proteins. Constitutive *CIS1* (tagged and untagged) also protected mtDNA during mitochondrial import stress caused by overexpression of *MGE1* and even partially suppressed the detrimental effects of deleting *PDR3* on mtDNA maintenance (Fig. 5I and fig. S4F). Thus, *CIS1* is an important effector of the mitoCPR. Cis1 reduces the levels of unimported proteins and protects mitochondrial functions during mitochondrial import stress.

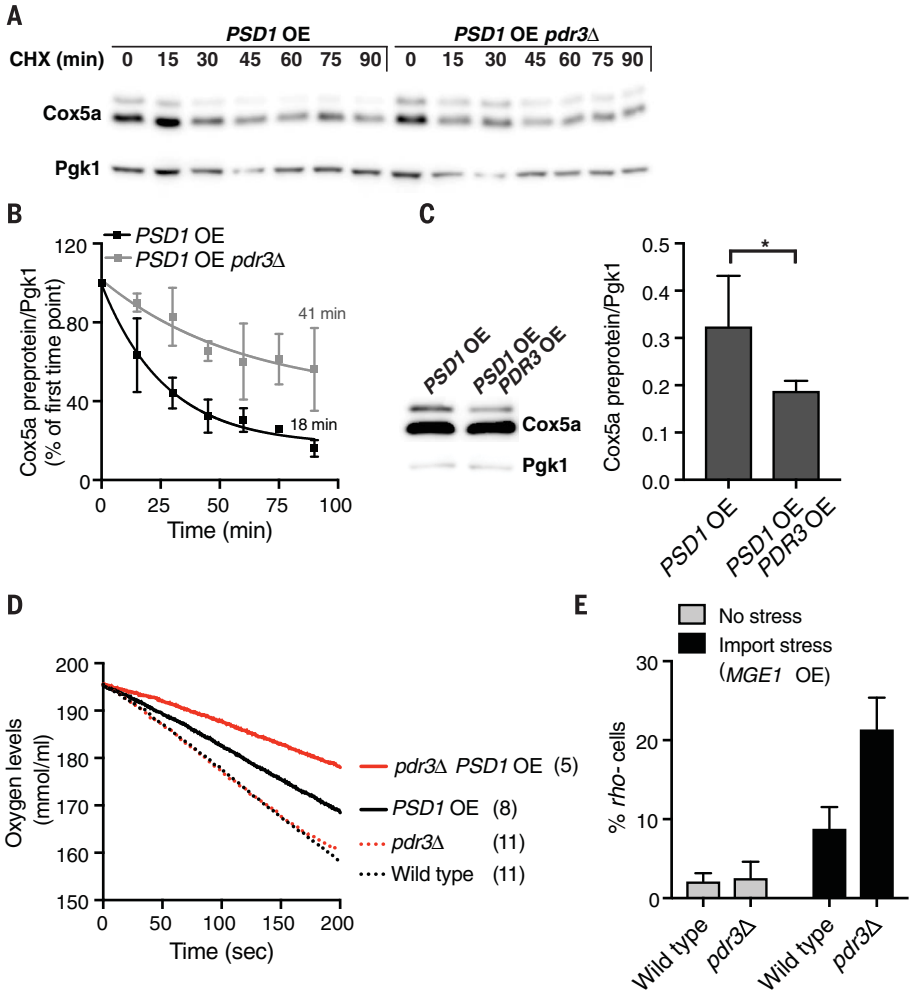

Fig. 4. The mitoCPR protects mitochondrial functions during import stress. (**A**) *PSD1* was overexpressed for 6 hours, and the half-life of Cox5a preprotein was examined after cycloheximide (0.5 mg/ml) addition in wild-type or *pdr3*Δ cells. CHX, cycloheximide; OE, overexpression. Pgk1 served as a loading control. (**B**) Quantification of (A); Cox5a preprotein half-life. *n* = 4 experiments; data are mean ± SD. (**C**) Immunoblot of Cox5a-V5 from *GAL-PSD1* cells or *GAL-PSD1* cells overexpressing *PDR3* (*TEF2-PDR3*) 6 hours after galactose induction. Quantification of Cox5a preprotein from three independent experiments is depicted on the right. Data are mean ± SD. Statistics were performed by using the Student's *t* test; **P* ≤ 0.05. (**D**) Oxygen consumption of wild-type and *pdr3*Δ cells that did or did not overexpress *PSD1* for 4 hours. The oxygen consumption rate (nmol s^{-1} ml) of this experiment is shown in parentheses. (**E**) *GAL-MGE1* and *GAL-MGE1 pdr3*Δ cells were grown for 24 hours in the presence or absence of galactose so as to induce *GAL-MGE1*. Mitochondrial DNA loss was analyzed by the appearance of *rho*$^-$ colonies on 1% yeast extract, 2% peptone (YEP) plates containing 2% ethanol and 0.3% glucose. *n* = 4 experiments; data are mean ± SD.

Cis1 and Msp1 mediate mitochondrial preprotein clearance during mitochondrial import stress

Our results indicate that during mitochondrial import stress, Cox5a^{pre} accumulated on the surface of mitochondria and appeared to be stalled in the translocase (Fig. 2, A and B). Cis1 aided in the import of preproteins, facilitated their degradation at the mitochondrial surface, or contributed to both. To test whether Cis1 promoted the degradation of unimported proteins, we asked whether down-regulation of Cox5a^{pre} brought about by constitutive *CIS1* expression depended on the proteasome. Although constitutive *CIS1* prevented the accumulation of Cox5a^{pre} in wild-type cells treated with CCCP (Fig. 5, F to H), it failed to do so in cells that carried the temperature-sensitive *rpn6-1* allele and thus had compromised proteasome function (Fig. 6, A and B). *MET25-COX5a* was likely induced before methionine depletion in the *rpn6-1* mutant because the transcription factor responsible for activating *MET25* is a proteasome substrate (*37*). Thus, *CIS1* promotes proteasomal degradation of unimported proteins that accumulate at the mitochondrial surface.

How does Cis1 promote the degradation of unimported proteins? The AAA–adenosine triphosphatase (ATPase) Msp1 is a dislocase that extracts endoplasmic reticulum (ER) and peroxisome membrane proteins mistargeted to the mitochondrial outer membrane for proteasomal degradation (*38*–*41*). Our results show that Msp1 has a similar function in reducing preprotein accumulation during mitochondrial import stress. Cells lacking *MSP1* accumulated high levels of Cox5a^{pre} when Cox5a expression was induced under conditions of mitochondrial import stress (CCCP treatment) (Fig. 6, C and D). Furthermore, accumulation of mature Cox5a was significantly delayed, suggesting that less Cox5a was imported into mitochondria (Fig. 6, C and E). Cells lacking *MSP1* neither accumulated Cox5a^{pre} nor induced mitoCPR under normal growth conditions (Fig. 6F and fig. S5A), excluding the possibility that *msp1*Δ cells were generally defective in importing proteins into mitochondria. An effect on Cox5a^{pre} was also observed when the *msp1-E193Q* allele was overexpressed from the *GAL1-10* promoter in cells lacking endogenous *MSP1* (Fig. 6, G and H, and fig. S5B). The *E193Q* substitution, located in the Walker B motif of the ATPase domain, is predicted to disrupt ATPase activity and stabilizes ER- and peroxisome-mistargeted proteins in the outer membrane of mitochondria (*38*–*40*). Thus,

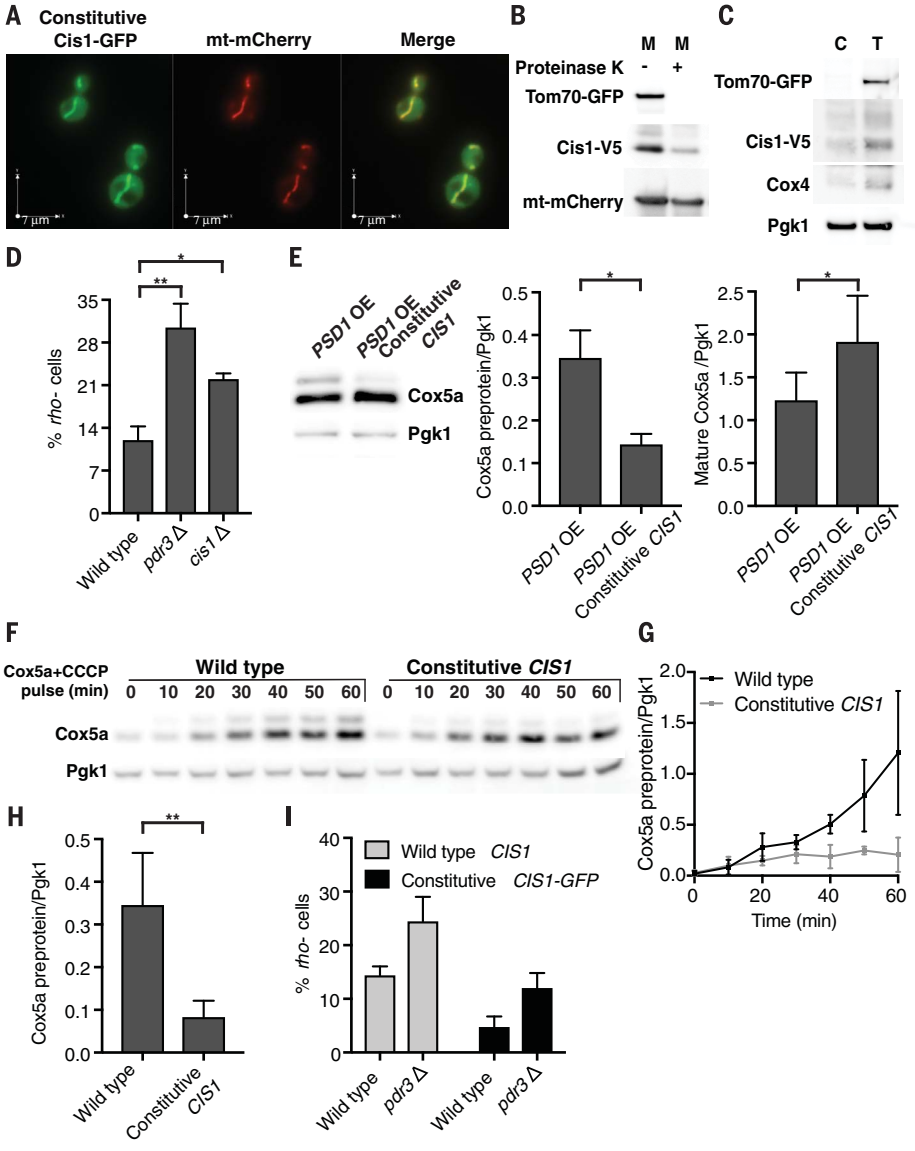

Fig. 5. Cis1 maintains mitochondrial function during protein import stress. (**A**) Live cell fluorescence imaging of cells expressing *TEF2-CIS1-GFP* and mitochondrial targeted mCherry (mt-mCherry). (**B**) Mitochondria were isolated from cells expressing *TEF2-CIS1-V5* that were grown in 3% glycerol. Mitochondria (M) (± proteinase K) are shown. mt-mCherry-matrix control protein, Tom70-GFP–outer membrane control protein. (**C**) Cytosolic fraction of cells presented in (B). Cytosolic (C) fraction as well as total cell lysate (T) are shown. Pgk1 served as a cytosol control protein, Tom70-GFP served as an outer-membrane control protein, and Cox4 served as a control matrix protein. (**D**) Wild-type, *pdr3*Δ, and *cis1*Δ cells were grown for 48 hours in the presence of galactose so as to induce *GAL-MGE1*. Mitochondrial DNA loss was analyzed through the appearance of *rho*$^{-}$ (petite) colonies. $n = 3$ experiments; data are mean ± SD. Student's *t* test was used; $^{*}P \le 0.05$, $^{**}P \le 0.005$. (**E**) Immunoblot analysis of Cox5a from *GAL-PSD1* or *GAL-PSD1 TEF2-CIS1* cells after *PSD1* overexpression (6 hours). OE, overexpression. Quantifications of Cox5a preprotein (middle) and mature Cox5a (right) are shown. $n = 3$ experiments; data are mean ± SD. Student's *t* test was used; $^{*}P \le 0.05$. (**F**) Wild-type or *TEF2-CIS1* cells were grown in the presence of methionine. *MET25-COX5a* was then induced through methionine removal in the presence of CCCP. Cox5a-V5 protein levels were analyzed at the indicated times (Pgk1, loading control). (**G**) Quantification of (F); Cox5a preprotein. $n = 4$ experiments; data are mean ± SD. (**H**) Quantification of Cox5a preprotein levels 60 min after induction of *MET25-COX5a* in the presence of CCCP. $n = 6$ experiments; data are mean ± SD. Student's *t* test was used; $^{**}P \le 0.005$. (**I**) Wild-type and *pdr3*Δ cells (± *TEF2-CIS1-GFP*) were grown for 24 hours in the presence of galactose so as to induce *GAL-MGE1*. Mitochondrial DNA loss was analyzed as in (D). $n = 4$ experiments; data are mean ± SD.

like Cis1, Msp1 limits the accumulation of unimported precursor proteins.

Next, we determined the epistatic relationship between *MSP1* and *CIS1*. We asked whether *CIS1*'s ability to limit the accumulation of Cox5a^{pre} required *MSP1*. Whereas *TEF2-CIS1* prevented the accumulation of Cox5a^{pre} in wild-type cells (Fig. 5, F and G), it failed to do so in cells lacking *MSP1* (Fig. 6, I and J). Thus, Cis1's effect on preprotein clearance depended on *MSP1*.

Having established that Cis1 and Msp1 both function in preprotein clearance during mitochondrial import stress, we next asked whether the two proteins act in the same pathway. Cis1 expressed from the *TEF2* promoter coimmunoprecipitated with Msp1-E193Q-FLAG, and vice versa (Fig. 7A and fig. S6A). We were not able to detect binding between Cis1 and wild-type Msp1 most likely because this interaction is transient (fig. S6B). We did, however, obtain genetic evidence to indicate that the two proteins interact. In cells lacking *GET1*, ER membrane proteins accumulate in the mitochondrial outer membrane (*38*, *39*). These conditions did not induce the mitoCPR but caused a growth defect at 37°C (fig. S5, A and C) (*38*). Overexpression of *CIS1*, like deletion of *MSP1*, enhanced this growth defect (fig. S5C), suggesting that high levels of Cis1 reduce the interaction of Msp1, with ER proteins mistargeted to the mitochondrial outer membrane.

The observation that the association of preproteins with mitochondrial membranes was resistant to sodium carbonate treatment suggested that preproteins accumulate at translocases during mitochondrial import stress (Fig. 2, B and C). We therefore asked whether Cis1 was also found at translocases. This appeared to be the case. Localization of Cis1 to mitochondria was dependent on Tom70, a receptor of the outer-membrane translocase (Fig. 7B). Furthermore, Cis1 interacted with Tom70 as well as with Msp1 as assessed with coimmunoprecipitation analysis (Fig. 7C).

Because Cis1 is only expressed during mitochondrial import stress (fig. S4A), we conclude that Cis1 is recruited to mitochondria under import stress, during which it interacts with both Tom70 and Msp1. Consistent with this conclusion, the interaction between Tom70 and Msp1 was enhanced during mitochondrial import stress (Fig. 7, D and E). We propose that upon recruitment to the translocase via Cis1, Msp1 evicts preproteins from the translocase and the mitochondrial surface to target them for proteasomal degradation. Our results do not exclude the possibility that Cis1 and Msp1 also improve import efficiency. We have some evidence to suggest that this may in fact be the case. Overexpression of *CIS1* caused an increase in mature Cox5a levels during prolonged mitochondrial import stress brought about by high levels of Psd1 (Fig. 5E). Similarly, *msp1*Δ cells accumulated less mature Cox5a after CCCP treatment (Fig. 6, C and E).

Discussion

Here, we describe the discovery of a surveillance mechanism, mitoCPR, that detects mitochondrial

import stress and protects mitochondrial functions in response. We propose that the mitoCPR effector Cis1 recruits Msp1 to the outer-membrane translocase to clear stalled proteins from the translocase, and consequentially improve mitochondrial import (Fig. 7F). This response is essential to protect mitochondrial functions and to maintain the mitochondrial genome during import stress. Recently, it was discovered that translation by ribosomes at the surface of mitochondria can stall (*42*). Whether the Msp1-Cis1 complex can clear preproteins from ribosomes during cotranslational import or whether the complex only recognizes posttranslationally imported proteins has yet to be determined. We also do not yet know whether Cis1 and Msp1 improve mitochondrial import solely by clearing unimported proteins. Our data suggest that they may also aid in the import process itself. Mitochondrial preproteins must be kept unfolded in order to translocate into mitochondria (*2*). A delay in mitochondrial import could result in premature folding and perhaps even aggregation of preproteins at the organelle's surface. We speculate that Msp1, whose ATPase domain faces the cytosol, could unfold prematurely folding or aggregated preproteins, giving them a second chance to translocate into mitochondria or, when this does not occur, target them for degradation (Fig. 7F).

The mitoCPR likely performs additional functions. Mitochondrial import defects lead to widespread mitochondrial dysfunction. Up-regulation of NADPH-dependent enzymes suggests a potential role for mitoCPR in restoring redox potential. Induction of genes involved in lipid metabolism argues for an effort to compensate for lipid biosynthesis disruption. Last, up-regulation of ABC transporter gene expression may be indicative of detoxification efforts aimed at removing toxic metabolic intermediates that could accumulate in the cytosol as a result of mitochondrial dysfunction.

We have not yet been able to identify the signal (or signals) that activates the mitoCPR. We can thus only speculate as to how the pathway is activated. In the MDR, Pdr1 and Pdr3 are activated by binding to xenobiotics (*43*). Mitochondrial dysfunction resulting from defects in mitochondrial import could lead to accumulation of metabolic intermediates in the cytoplasm, which in turn bind to and activate Pdr3. It is also possible that specific unimported proteins activate Pdr3. Such mechanisms have been described for the mitochondrial unfolded protein response and the recognition of damaged mitochondria in mammals (*44*, *45*).

We have studied the mitoCPR in response to overexpression of bipartite signal–containing proteins. Although this is unlikely to occur under physiological conditions, budding yeast cells are exposed to microorganisms that produce compounds known to interfere with mitochondrial import in the wild (*35*). Import defects could also result from disease or mitochondrial stress conditions such as high levels of reactive oxygen species. *CIS1* and other mitoCPR genes are induced during diauxic shift, a physiological state defined as the switch from glycolysis to respiration that occurs when fermentable carbon sources become limiting (*46*). Switch to respiratory growth requires an expansion of the mitochondria compartment. We propose that this increase in mitochondrial

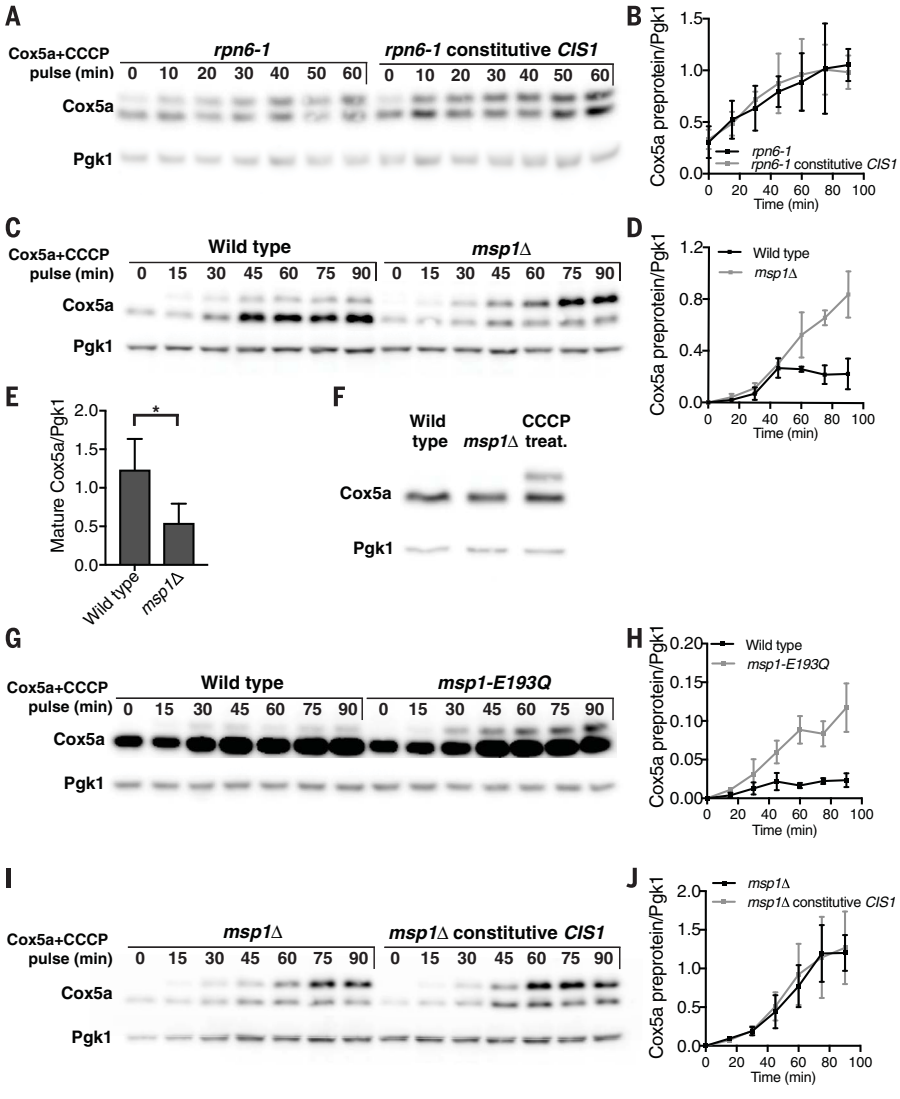

Fig. 6. Cis1 and Msp1 are required for preprotein clearance after mitochondrial import stress. (**A**) *rpn6-1* or *rpn6-1 TEF2-CIS1* cells were grown at room temperature in the presence of methionine. Cells were then transferred into medium lacking methionine with 20 μM CCCP at 30°C. The accumulation of Cox5a-V5 preprotein (encoded by *MET25-COX5a*) is shown. (**B**) Quantification of (A); Cox5a preprotein levels from four independent experiments. Data are mean ± SD. (**C**) Wild-type or *msp1*Δ cells were grown at 30°C with methionine and treated as in (A). (**D**) Quantification of (C); Cox5a preprotein levels from four independent experiments. Data are mean ± SD. (**E**) Quantification of (C); Mature Cox5a levels 60 min after induction. $n = 4$ experiments; data are mean ± SD. Statistics were determined by using the Student's t test. $^*P \leq 0.05$. (**F**) Immunoblot analysis of Cox5a-V5 from wild-type cells, wild-type cells treated with 20 μM CCCP for 1 hour, or *msp1*Δ cells. (**G**) Wild-type cells or cells expressing *msp1-E193Q* from the inducible *GAL1-10* promoter were grown in the presence of galactose for 6 hours. Cells were then transferred to medium lacking methionine and containing 20 μM CCCP, and the accumulation of inducible Cox5a-V5 preprotein (encoded by *MET25-COX5a*) was examined. Cox5a levels were higher in this experiment because *MET25-COX5a* expression is higher in medium containing raffinose/galactose than in glucose (fig. S5B). (**H**) Quantification of (G); Cox5a preprotein levels from three independent experiments. Data are mean ± SD. (**I**) *msp1*Δ cells or *msp1*Δ cells expressing *TEF2-CIS1* were treated as in (C). The experiment shown in (C) was performed in parallel, and results can thus be directly compared. (**J**) Quantification of (I); Cox5a preprotein levels from three independent experiments. Data are mean ± SD.

mass, which requires increased mitochondrial import, leads to mitochondrial import stress. Mitochondria of multicellular eukaryotes are less likely to be exposed to mitochondrial poisons in the environment but do undergo increased biogenesis in specific tissues and during development. Whether a mitochondrial import stress response exists in higher eukaryotes has yet to be determined.

Materials and methods

Yeast strains and growth conditions

All strains are derivatives of W303 (AA2587) and are listed in table S2. Cells were grown overnight in YPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C to saturation, then diluted in fresh YPD (OD600 = 0.1) and grown until they reached logarithmic phase. To induce the *GAL1-10* promoter, cells were grown overnight at 30°C in minimal selective medium containing 2% raffinose or in YPR (1% yeast extract, 2% peptone, 2% raffinose). Cells were then diluted to OD = 0.3 or OD = 0.1 and recovered for an hour or 3 hours, respectively, following the addition of galactose to a final concentration of 1% for 4 hours (for measuring mRNA levels) or 6 hours (for protein analysis). To induce *MET25-COX5a*, cells were grown overnight in YPD supplemented with 8 mM methionine. Cells were diluted to OD = 0.1, grown for a few hours and then switched to medium lacking methionine [Complete supplement mixture w/o methionine (CSM, MP Biomedicals), yeast nitrogen base w/o amino acids (Difco), 2% glucose, titered to pH 7]. CCCP was added to a final concentration of 20 μM.

Wild-type cells were incubated in the presence of 5 μg/ml ethidium bromide in YPD for 72 hours to obtain *rho0* cells. *rho0* state was verified by DAPI staining. *rho*$^-$ cells were obtained by deletion of the mitochondrial ribosomal subunit *MRPL16* (*47*). The *mrpl16*Δ strain was confirmed to be *rho*$^-$ by its inability to grow on medium lacking a fermentable carbon source as a haploid and as a diploid following mating with *rho0* cells. The presence of mitochondrial DNA in *mrpl16*Δ cells was tested by DAPI.

The plasmid pRS426 was used as an empty plasmid control. A plasmid expressing mt-mCherry and integrated into the *LEU2* locus was cloned from plasmid pHS12-mCherry (a gift from Benjamin Glick, Addgene plasmid # 25444).

Immunoblot analysis

For immunoblot analyses, ~2 OD_{600} units of cells were harvested and treated with 5% trichloroacetic acid overnight at 4°C. The acid was washed away with acetone and the cell pellet was subsequently dried. The cell pellet was pulverized with glass beads in 100 μl of lysis buffer (50 mM Tris–HCl at pH 7.5, 1 mM EDTA, 2.75 mM DTT) using a bead-beater. 3 × SDS sample buffer was added and the cell homogenates were boiled. Samples were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and subsequently incubated with anti-V5 antibodies (1:2000 dilution; Life Technologies), anti-3-PhosphoGlycerate Kinase antibodies (1:5000 dilution; Invitrogen), anti-GFP antibodies (1:1000; Clontech, JL-8), anti-Kar2 (1:200,000 dilution; kindly provided by Mark Rose), anti-Myc antibodies (1:1000 dilution; Sigma, 9E10), anti-Cox4 antibodies (1:1000; Abcam) or anti-FLAG antibodies (1:1000; Sigma). HRP-linked sheep anti-mouse antibodies and

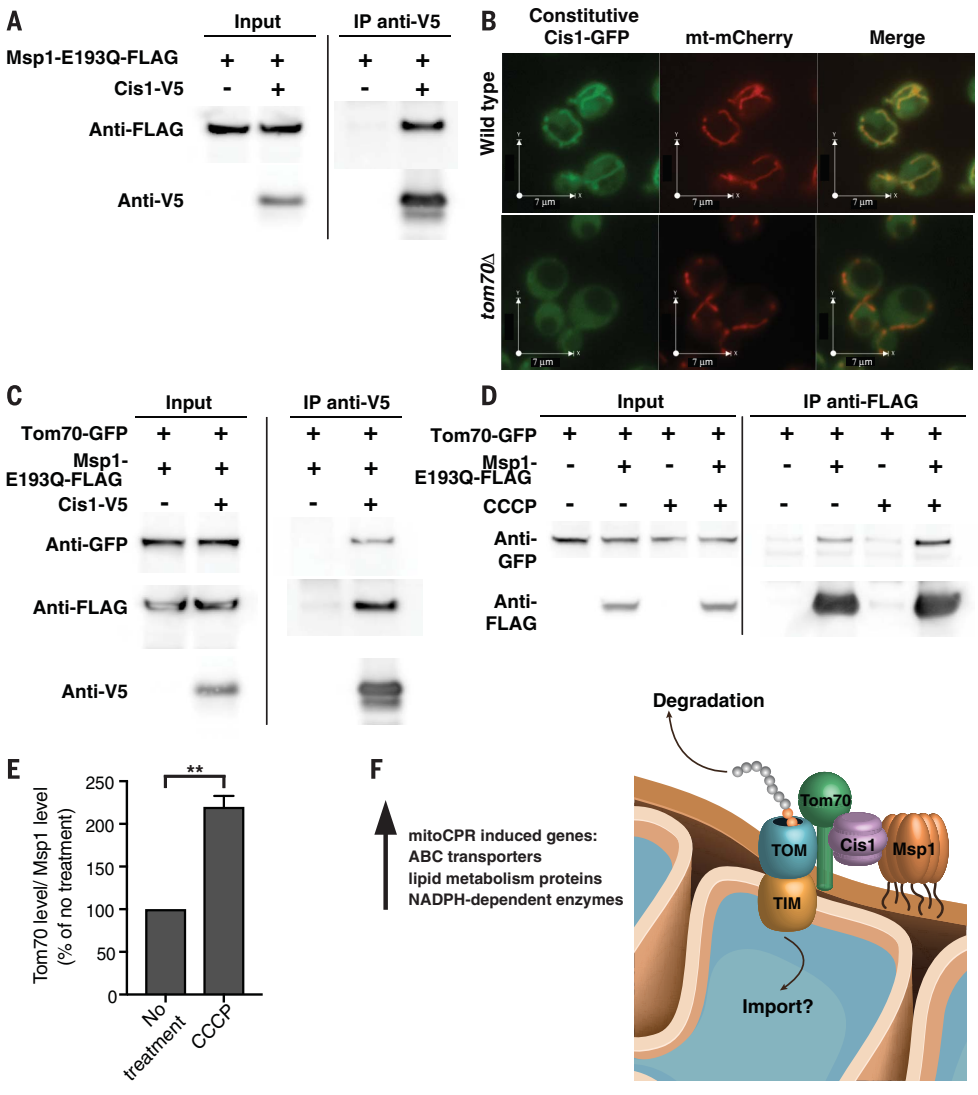

Fig. 7. Cis1 interacts with Msp1 and with the outer-membrane translocase. (**A**) Cells expressing *msp1-E193Q-FLAG* and cells expressing *msp1-E193Q-FLAG* and *TEF2-CIS1-V5* were grown in yeast extract, peptone, and glucose (YPD). Cells were lysed, and Cis1-V5 was immunoprecipitated by using antibodies to V5. (**B**) Live cell fluorescence imaging of wild-type or *tom70Δ* cells expressing *TEF2-CIS1-GFP* and mitochondrial-targeted mCherry (mt-mCherry). (**C**) Cis1-V5 (encoded by *TEF2-CIS1-V5*) was immunoprecipitated by using antibodies to V5 from *TOM70-GFP*– and *msp1-E193Q-FLAG*–expressing cells. Cells expressing only *TOM70-GFP* and *msp1-E193Q-FLAG* were used as control. (**D**) Cells expressing *TOM70-GFP* or *msp1-E193Q-FLAG* and *TOM70-GFP* were grown in YPD in the presence or absence of 20 μM CCCP for 1 hour. Msp1-E193Q-FLAG was immunoprecipitated by using antibodies to FLAG. (**E**) Quantification of (D); coimmunoprecipitated Tom70 levels (normalized to coimmunoprecipitated Msp1 levels) in nontreated and CCCP-treated cells from three independent experiments. No treatment was set to 100%. Data are mean ± SD. Statistics were performed by using the Student's *t* test; $^{**}P \leq 0.005$. (**F**) A model for how Cis1 and Msp1 affect mitochondrial import during import stress. IMS, intermembrane space.

HRP-linked donkey anti-rabbit antibodies (GE Healthcare) were used as secondary antibodies. Statistics were performed using the Student's *t* test. The protein half-life in Fig. 4B was analyzed as a one-phase exponential decay chart using Prism software.

Fluorescence microscopy

Cells were grown overnight in minimal medium at 30°C, diluted to OD = 0.1 and grown to logarithmic phase. Images were acquired with a DeltaVision Elite microscope (GE Healthcare Bio-Sciences, Pittsburgh, PA). Images were taken with a 100× plan-Apo objective, an InsightSSI solid-state light source, and a CoolSNAP HQ2 camera.

Real-time PCR

Total RNA was isolated using the RNeasy mini-kit (Qiagen). RNA (750 ng) was used to generate cDNAs using the SuperScript III first strand synthesis system (Life Technologies). Quantitative PCR was performed using a SYBR green mix (Life Technologies) and amplified using a LightCycler 480 II (Roche). Signals were normalized to *ACT1* transcript levels and are presented as fold increase of control conditions.

Gene expression analysis

For RNA expression analysis, *PSD1* was overexpressed for 4 hours. Total yeast RNA was isolated using the RNeasy mini-kit (Qiagen) and samples were sequenced on a HiSeq 2000. *S. cerevisiae* RNA-seq reads were aligned to the sacCer3 genome with STAR version 2.5.3a and Ensembl transcripts were quantified using rsem version 1.3.0. Differential expression analysis was performed using deseq2 version 1.16.1 running under R version 3.4.0. Default options were selected for deseq2 runs except cooksCutoff and independent Filtering were both set to false during results preparation and unmoderated fold changes were used. RNA sequencing data can be accessed via the following link: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107784.

Mitochondrial oxygen consumption

Cells were grown overnight at 30°C in minimal selective medium with 2% raffinose. The cells were then diluted to OD = 0.3 and recovered for an hour following the addition of galactose to a final concentration of 1% for 4 hours. Cells were then transferred to YPG (1% yeast extract, 2% peptone, 3% glycerol) and incubated for 20 min. Oxygen consumption rate was measured from 0.75 OD (1 ml) cells in YPG using an Oxytherm instrument (Hansatech) for 3 min at 25°C. The slope of the linear range of oxygen depletion was used to measure oxygen consumption rate of 3 experiments. Statistics were performed using the Student's *t* test.

Mitochondrial DNA maintenance assay

The analysis of mtDNA maintenance was described previously (*48*). Cells were grown overnight at 30°C in minimal selective medium with 2% glucose. Cells were then diluted to OD = 0.15 in minimal selective medium with 2% raffinose and were grown for 3 hours following the addition of galactose to a final concentration of 1% for 24 or 48 hours. Within these 24 hours (8 hours after induction) the cells were diluted 1:20 into the same medium. Yeast cells (~200) were spread on plates containing 1% yeast extract, 2% peptone, 0.3% glucose, 2% ethanol and were grown at 30°C for 3 days until all colonies could be detected. The percentage of small *rho*$^-$ (petite) colonies was determined from 3 different experiments.

Membrane potential measurements

Cells lacking *PDR1*, *PDR3*, and *PDR5* (to prevent efflux of dyes out of the cells) bearing either an empty plasmid (for control and CCCP treatment) or a *GAL-PSD1* containing plasmid and expressing a mitochondria-targeted mCherry (mt-mCherry) were grown overnight at 30°C in minimal selective medium containing 2% raffinose. The cells were diluted to OD = 0.3 and recovered for an hour following the addition of galactose to a final concentration of 1% for 4 hours. CCCP (20 μM) was added for 1 hour. Cells were then transferred to 1 ml dye buffer (10 mM Hepes pH 7.2 and 5% glucose) and incubated with 2.5 μM Rhodamine 123 (Thermo Fisher Scientific) for 15 min at room temperature. Cells were washed 5 times in 1.5 ml dye buffer. Mitochondria were identified by mt-mCherry labeling. Membrane potential was analyzed by the following equation: (mitochondrial fluorescence intensity – cytosolic fluorescence intensity)/cytosolic fluorescence intensity cytosol.

Mitochondria isolation

Cells were grown to logarithmic phase, collected by centrifugation and washed once with water. Cells were then resuspended in 0.1 M Tris pH 9.4, 10 mM DTT and incubated for 20 min at 30°C. Cell walls were disturbed by incubation in 1.2 M sorbitol, 20 mM K_2HPO_4 pH 7.4, 1% zymolyase for 1 hour at 30°C. Dounce homogenization was used to lyse the cells in 0.6 M sorbitol, 10 mM Tris pH 7.4, 1 mM EDTA, fatty acid free 0.2% BSA and 1 mM PMSF. Mitochondria were then isolated by differential centrifugation as described previously (*49*) and resuspended in SEM buffer (0.25 M sucrose, 10 mM MOPS KOH pH 7.2 and 1 mM EDTA). Proteinase K was added to a final concentration of 50 μg/ml for 5 min at 37°C and the reaction was stopped by the addition of 4 mM PMSF for 15 min on ice.

For sodium carbonate extraction, 40 μg of mitochondria were pelleted and resuspended in 500 μl of 100 mM sodium carbonate pH 11 or in SEM buffer for the untreated control. The samples were kept on ice for 30 min followed by centrifugation at 90,000 g for 30 min. Supernatants and pellets were incubated with 12.5% TCA overnight at 4°C and separated by SDS-PAGE.

Coimmunoprecipitation assays

Cells were grown in YPD to OD = 0.9 when not treated or to OD = 0.7 following treatment with 20 μM CCCP for 1 hour. Approximately 50 OD units of cells were collected, washed once with water and frozen. Cells were lysed with Silica Beads using a FastPrep instrument (speed 6.5, 45 s, 3 cycles) with 200 μl IGEPAL buffer [50 mM Tris pH 7.5, 150 mM NaCl, 1% IGEPAL and Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific)]. Lysates were brought up to 1.5 ml with IGEPAL buffer containing 0.2% BSA. Lysates were clarified by centrifugation at 20,000 g for 10 min at 4°C. Twenty μl of Anti-V5 agarose affinity gel antibody (Sigma) or Anti-FLAG M2 affinity gel (Sigma) were added and lysates were incubated for 2 hours at 4°C. Beads were then washed 5 times with IGEPAL buffer containing 0.2% BSA. Sample buffer was added to the beads, which were then boiled. Final eluates and two percent of the lysates were separated by means of SDS-PAGE.

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S6
Tables S1 and S2

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MitoCPR—A surveillance pathway that protects mitochondria in response to protein import stress

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The mitoCPR unclogs mitochondria

The import of proteins into mitochondria is essential for cell viability. How cells respond when mitochondrial protein import is impaired is poorly understood. Weidberg and Amon showed that upon mitochondrial import stress, yeast cells mounted a response known as the mitoCPR. mitoCPR was activated when mitochondrial protein import was impaired and unimported precursors accumulated on the organelle's surface. mitoCPR restored mitochondrial functions by clearing stalled proteins from the import channels. It did this by inducing expression of Cis1, which recruited the adenosine triphosphatase Msp1 to import channels to remove unimported precursors and target them for degradation by the proteasome.

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