Retro-translocation of mitochondrial intermembrane space proteins

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The content of mitochondrial proteome is maintained through two highly dynamic processes, the influx of newly synthesized proteins from the cytosol and the protein degradation. Mitochondrial proteins are targeted to the intermembrane space by the mitochondrial intermembrane space assembly pathway that couples their import and oxidative folding. The folding trap was proposed to be a driving mechanism for the mitochondrial accumulation of these proteins. Whether the reverse movement of unfolded proteins to the cytosol occurs across the intact outer membrane is unknown. We found that reduced, conformationally destabilized proteins are released from mitochondria in a size-limited manner. We identified the general import pore protein Tom40 as an escape gate. We propose that the mitochondrial proteome is not only regulated by the import and degradation of proteins but also by their retro-translocation to the external cytosolic location. Thus, protein release is a mechanism that contributes to the mitochondrial proteome surveillance.

Mitochondrial biogenesis is essential for eukaryotic cells. Because most mitochondrial proteins originate in the cytosol, mitochondria had to develop a protein import system. Given the complex architecture of these organelles, with two membranes and two aqueous compartments, protein import and sorting require the cooperation of several pathways. The main entry gate for precursor proteins is the translocase of the outer mitochondrial membrane (TOM) complex. Upon entering mitochondria, proteins are routed to different sorting machineries (1–5).

Reaching the final location is one step in the maturation of mitochondrial proteins that must be accompanied by their proper folding. The mitochondrial intermembrane space assembly (MIA) pathway for intermembrane space (IMS) proteins illustrates the importance of coupling these processes because this pathway links protein import with oxidative folding (6–10). Upon protein synthesis in the cytosol, the cysteine residues of IMS proteins remain in a reduced state, owing to the reducing properties of the cytosolic environment (11, 12). After entering the TOM channel, precursor proteins are specifically recognized by Mia40 protein, and their cysteine residues are oxidized through the cooperative action of Mia40 and Erv1 proteins (7, 13–17). Mia40 is a receptor, folding catalyst, and disulfide carrier, and the Erv1 protein serves as a sulfhydryl oxidase. The oxidative folding is believed to provide a trapping mechanism that prevents the escape of proteins from the IMS back to the cytosol (10, 13, 18). Our initial result raised a possibility that the reverse process can also occur, as we observed the relocation of in vitro imported Tim8 from mitochondria to the incubation buffer (13). Thus, we sought to establish whether and how this process can proceed in the presence of the intact outer membrane (OM). Our study provides, to our knowledge, the first characterization of the mitochondrial protein retro-translocation. The protein retro-translocation serves as a regulatory and quality control mechanism for the mitochondrial IMS proteome.
form (i-form) cleaved by the mitochondrial processing peptidase MPP and the soluble mature form (m-form) additionally cleaved by the IM peptidase IMP (20). In such an experimental setup, inward transport no longer depends on MIA-driven oxidative folding, and backward movement from mitochondria could be dissected. The processing of b₂-Mia₄₀_core and its four single cysteine-to-serine variants was monitored in cellular protein extracts (Fig. 1A). Two forms of the fusion protein, i-form and m-form, were detected. The mutant of b₂-Mia₄₀_core, in which the third cysteine residue was replaced by serine (C₃S), was an exception because this protein is likely unable to fold properly (21). No accumulation of the m-form was observed, whereas the i-form of b₂-Mia₄₀_core-C₃S accumulated at levels similar to other b₂-Mia₄₀_core variants (Fig. 1A, lane 5). This indicates specific disappearance of m-b₂-Mia₄₀_core-C₃S. When wild-type b₂-Mia₄₀_core was expressed in the strain with impaired oxidative folding (erv1-2int), the m-form was not accumulated, mimicking the behavior of b₂-Mia₄₀_core-C₃S (Fig. 1B). Similarly, when the radiolabeled b₂-Mia₄₀_core precursor was imported into isolated mitochondria in the presence of iodoacetamide (IA), which inhibits oxidative folding by blocking thiol groups, the decrease in the m-form but not i-form was observed (Fig. 1C, Top). As no effect of the IA treatment on the import and processing of MIA-unrelated b₂ fusion proteins was observed (Fig. 1C, Bottom, and Fig. S1A), we concluded that inhibition of oxidative folding by removing or blocking thiol groups resulted in the inefficient maintenance of proteins in the IMS.

The depletion of m-b₂-Mia₄₀_core-C₃S could be caused either by IMS proteases (22) or backward movement to the cytosol for proteasomal degradation (23). The m-b₂-Mia₄₀_core-C₃S form was not rescued by the deletion of any of the known yeast IMS proteases (i.e., Yme1, Atp23, and Prd1; Fig. S1B). Because the cleavage by IMP exposes glutamic acid residue at the N terminus of m-b₂-Mia₄₀_core-C₃S, we tested strains that are defective in the cystolic Arg/N-terminal glutamic acid residue requires arginine conjugation (Fig. 1D) (24). To enter the Arg/N-end rule pathway, the N-terminal glutamic acid residue requires arginine conjugation by the arginyl transferase Ate1. In Δate1 cells, the increased accumulation of the m-b₂-Mia₄₀_core-C₃S m-form was observed (Fig. 1D, lane 4). With deletion of the E2 ubiquitin-conjugating enzyme Rad6, the accumulation of a band of slightly slower migration was visible (Fig. 1D, lane 3), which indicated possible arginylation by Ate1 preceding ubiquitination by the Ubr1/Rad6 heterodimer and degradation (24, 25). The m-b₂-Mia₄₀_core-C₃S rescue in the Δrad6 and Δate1 cells indicated exposure of the m-form to the cytosolic quality control machinery supporting the release hypothesis. This process was incomplete owing to a possible contribution of other degradation mechanisms.

**Disulfide Bond Reduction Causes Protein Escape from the IMS.** We established an *in organello* assay for the release of IMS proteins. The isolated mitochondria were treated with the reducing agent DTT to allow the reduction of disulfide bonds (Fig. 2A). The MIA substrate proteins Cox12, Cox17, and Pet191 were found to be decreased in the mitochondria and increased in the release fraction (Fig. 2A, lanes 1 and 2 vs. 3 and 4). The OM integrity was unaffected by DTT because the control IMS-soluble and MIA-independent proteins (Ccp1, Cyc1, and Mpm1) remained unaffected (Fig. 2A), whereas they were efficiently released upon hypoosmotic swelling (Fig. S2A, lanes 1–4). In addition, the OM integrity was demonstrated by treatment with the protease. Proteins, which were sensitive to protease treatment upon swelling of mitochondria (Cox11, Cyc3, Mia₄₀, Mpm1, and Tim23; Fig. S2A and B), remained protease-protected (Fig. 2A, lanes 5 and 6 vs. 1 and 2). The OM protein Tom22 was digested as expected (Fig. 2A). Thus, the release of proteins was not due to a compromised integrity of mitochondrial membranes.

The mitochondrial and release fractions were treated with an agent that reacts with free thiol groups causing the migration shift (Fig. S3A). The released Cox12, Pet191, and also Erv1 proteins were found exclusively in the reduced state (i.e., modified by AMS) in contrast to their more oxidized state observed in the mitochondria (Fig. 2B, lane 8 vs. 5). In the case of Pet191, the relocation was almost complete, whereas Cox12 and Erv1 were partially released (Fig. 2B, lane 4 vs. 2 and 8 vs. 6). The unreleased remainder of Cox12 and Erv1 in the mitochondrial fraction was partially reduced (Fig. 2B, lane 6). As expected, the DTT treatment also affected the redox state of the membrane-anchored Mia₄₀ protein, whereas Mdj1 and Tom40 remained unaffected (Fig. 2B). A similar approach was used to test release susceptibility of small Tim proteins, a distinct class of MIA substrates. Tim8, Tim10, and Tim13 were found largely unaffected by the DTT treatment (Fig. S3B). This is in agreement with the

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**Fig. 1.** Proteins that are defective in oxidative folding can be released from the IMS to the cytosol. (A) Schematic representation and cellular levels of b₂-Mia₄₀_core variants. (B) Cellular levels of b₂-Mia₄₀_core expressed in WT and erv1-2int strains. (C) Import of [³⁵S]b₂-Mia₄₀_core and [³⁵S]b₂(167)-DHFR into WT mitochondria with or without IA (50 mM). (D) Cellular levels of b₂-Mia₄₀_core-C₃S in WT, Δrad6, and Δate1 strains. Proteins were analyzed by SDS/PAGE and immunodetection (A, B, and D) or autoradiography (C). i, intermediate; m, mature; p, precursor; WT, wild type; *, altered migration of b₂-Mia₄₀_core-C₃S m-form.
observation that mature small Tim proteins, which are assembled in heterohexameric complexes, are resistant to reducing agents (13, 26).

Next, we used an in organello release assay to confirm the release of the b2-Mia40core m-form (Fig. 2C). The b2-Mia40core-C4S variant was used because the accumulation of its m-form was similar to wild-type protein (Fig. 1A), but it was likely more prone to reduction. The m-b2-Mia40core-C4S form was efficiently released similarly to Cox12, Mix17, and Pet191 (Fig. 2C). Mitochondria were pretreated with protease to exclude release of proteins, which due to incomplete import might have accumulated in transit—that is, at the OM. The comparison of samples with and without protease pretreatment excluded such a scenario (Fig. 2C, lanes 1–4 vs. 5–8). The in organello assay was also applied to mitochondria isolated from a human cell line (HEK293). The human MIA substrate protein COX6B was released from mitochondria and recovered in the release buffer (Fig. 2D; for analysis of control proteins, see Fig. S4A and B). Thus, protein release is also a feature of human mitochondria.

The temperature-sensitive variant of Erv1 protein Erv1-2 (27) was accumulated in the cells to higher levels than wild-type Erv1 under permissive conditions (Fig. 3A). Upon shifting to the restrictive temperature, the Erv1-2 levels dropped significantly (Fig. 3A). To test whether this effect was connected with release, mitochondria were subjected to incubation at various temperatures (Fig. 3B). During incubation at the restrictive temperature (37 °C), Erv1-2 was found to be processed and its degradation product was partially released from mitochondria (Fig. 3B, lanes 7 and 10). In this case, the increase in temperature, without reducing treatment, was sufficient to cause destabilization of the mutant protein, accompanied by the release of its small degradation derivative.

Small Proteins Escape Mitochondria More Efficiently via the Tom40 Channel. To analyze the impact of protein size on its ability to escape mitochondria, a tandem head-to-tail fusion of two Pet191 molecules and FLAG tag was created (Pet191_Pet191FLAG), the molecular mass of which was doubled. The tandem protein was released from mitochondria less efficiently than the corresponding monomer Pet191FLAG protein (Fig. 4A). Pet191 contains an odd number of cysteine residues, at least one of which is likely reduced and thus should be available for thiol-modifying agents. This property of Pet191 allowed modification with methoxypolyethylene glycol maleimide (mPEG) (5,000 Da), adding both molecular mass and structural complexity through the formation of a side chain. The release of the modified protein was almost completely abolished, in contrast to Cox12 and Mix17 proteins, which did not...
undergo modifications under these conditions (Fig. 4B). These results demonstrate that both linear protein length and 3D complexity are among the limiting factors for protein retro-translocation.

The release of Cox12 upon DTT was decreased in the mitochondria isolated from Δtom5 compared with the wild-type cells (Fig. 4C; see Fig. S5A for mitochondrial samples). This raised the possibility that the TOM complex is responsible for the backward transfer of proteins. First, we excluded a direct effect of DTT on the cysteine residues of Tom40 by comparing the release from mitochondria with wild-type and cysteine-free variants of Tom40 (Fig. S5B). No difference in release efficiency was observed between Tom40CFREE and wild-type mitochondria (Fig. S5B). Next, to test the involvement of Tom40 in the release process, the Tom40C130/C138 variant was used, in which cysteine residues were placed in positions 130 and 138 (28) (Fig. S5C). The steady-state levels of mitochondrial proteins were not altered (Fig. S5D). The thiol groups of the engineered cysteine residues faced the lumen of Tom40 (Fig. S5C). This allowed for modification of Tom40C130/C138 with thiol-reacting agents in the intact mitochondria (Fig. 4D). Upon modification with mPEG (5,000 Da), spatial blockade was generated in the lumen of the Tom40C130/C138 channel that was recently demonstrated by us to inhibit protein import into mitochondria (29). Thus, we pretreated mitochondria with mPEG before the in organello release assay. The release of Cox12 and Mix17 from mitochondria with Tom40C130/C138 modified by mPEG was decreased compared with wild type (Fig. 4E). The efficient modification of Tom40C130/C138 in this experiment was confirmed by analysis of mitochondrial fractions (Fig. S5E). The Mix17FLAG fusion protein derivatives, which are present in mitochondria (Fig. S5F), were also released less efficiently upon channel blockade (Fig. 4F; see Fig. S5G for mitochondrial protein release is size dependent and involves the TOM complex. (A) Release of Pet191FLAG and Pet191_Pet191FLAG from mitochondria upon treatment with DTT. (B) Release of Pet191 upon modification with mPEG (5,000 Da) and quantification. Signals from mitochondrial fractions not treated with DTT were set to 1. (C) Protein release from WT and Δtom5 mitochondria upon treatment with DTT and quantification. Signals from the mitochondrial fractions of WT and Δtom5 not treated with DTT were set to 1. Mitochondria fractions are shown in Fig. S5A. Mean ± SEM (n = 3). (D) Susceptibility of Tom40 and Tom40C130/C138 to thiol-modifying agents (AMS or mPEG) under native conditions. (E) Protein release from WT and Tom40C130/C138 mitochondria pretreated with mPEG upon treatment with DTT. Mitochondria fractions are shown in Fig. S5E. (F) Mix17FLAG release from Tom40C130/C138 mitochondria, with or without pretreatment with mPEG, upon treatment with DTT and quantification. The signal from the sample not treated with mPEG and incubated with DTT for 25 min was set to 1. Signals from DTT untreated samples were subtracted. Mean ± SEM (n = 3). Mitochondria fractions are shown in Fig. S5G. (A–F) Proteins were analyzed by SDS/PAGE and immunodetection. WT, wild type; *, full-size Mix17FLAG; **, Mix17FLAG degradation product.
samples). These results show the involvement of the Tom40 channel protein in the mitochondrial protein release.

**Protein Release Contributes to Mitochondrial Protein Quality Control.** We sought to determine whether protein release is also important for cell physiology. The cells that were grown in respiratory medium were transferred to fermentative media. Under these conditions, mitochondrial biogenesis slows because oxidative phosphorylation is not needed. The redox environment in the cytosol has been reported to be more reducing in fermentation than during respiration (30), likely affecting the environment of the IMS (31). The in vivo analysis of protein steady-state levels revealed a decrease in MIA substrate proteins (i.e., Cox12, Cox17, Mix17, and Pet191) upon both inhibition of protein synthesis with cycloheximide (CHX) under respiratory conditions or transfer to glucose (Fig. 5A). Additionally, the decrease in protein levels (Cox12, Cox17, and Pet191) was faster upon transfer to glucose, and the protein levels were significantly reduced (approx. 50% or less) only after 15 min (Fig. 5A, lanes 6–10). Cox12 and Pet191 were properly localized in mitochondria, similar to other mitochondrial proteins Mia40, Aco1, and Atp5 but in contrast to the cytosolic proteins Pgk1, Rpl17a, and Rpt5 (Fig. 5B). These observations indicated an active mechanism engaged in IMS protein removal during the switch from respiration to fermentation. We assessed whether Cox12 was accessible to the proteasome upon a shift to fermentation. Treatment with MG132 partially rescued the decrease in Cox12 (Fig. 5C). Similar stabilization was achieved in the presence of another proteasome inhibitor, PS341 (Fig. S6A). Thus, Cox12 was accessible to the ubiquitin-proteasome system in the cytosol. Next, we transformed wild-type cells with plasmids encoding FLAG-tagged MIA substrate proteins (Cox12FLAG or Pet191FLAG) under the control of a galactose-inducible promoter (23) to exclude a possibility that only newly synthesized proteins are substrates for the proteasome. The expression induced by galactose under respiratory conditions followed by the expression shutdown due to galactose removal was performed to minimize an effect of the new synthesis of ectopically expressed proteins (Fig. S6B, T1 vs. T0). Subsequently, cells were shifted to fermentation with or without the proteasome inhibitor. The levels of accumulated fusion proteins and their native counterparts were decreased upon a shift to fermentation and were rescued by proteasomal inhibition (Fig. S6B). These observations support the involvement of proteasomal degradation in the clearance of mature proteins that were released from mitochondria.

Fig. 5. Respiration-to-fermentation shift induces protein escape to the cytosol and proteasomal clearance. (A) Cellular protein levels upon inhibition of protein synthesis or shift from respiration to fermentation. Experimental scheme and quantification of Cox12 are provided. Signals from time point 0 were set to 1. (B) Subcellular localization of MIA substrate proteins in the cells grown under respiratory conditions. (C) Protein levels upon shift from respiration to fermentation with or without MG132 treatment. Experimental scheme and quantification of Cox12 are provided. Signals from time point 0 were set to 1. Mean ± SEM (n = 7). (D) Protein levels in WT and Δcox12 cells with plasmid expressing b2-Cox12FLAG upon shift from respiration to fermentation with or without MG132 treatment. Experiment was done based on the scheme as in C. (A–D) Proteins were analyzed by SDS/PAGE and immunodetection. CHX, cycloheximide; ev, empty vector; i, intermediate; m, mature; P, mitochondrial pellet; S, postmitochondrial supernatant, T, total; WT, wild type.
Finally, the fusion protein of Cox12FLAG with the cytochrome b2 presequence was expressed in the strain lacking COX2. The b2-Cox12FLAG was functional because it complemented the ΔDtot mutation and rescued upon proteasomal inhibition (Fig. 5D). However, the m-form was strongly decreased upon fermentation and rescue upon proteasomal inhibition (Fig. 5D), indicating that the mitochondrially processed and IMS-soluble m-form of protein was released from mitochondria and cleared by the proteasome. These experiments show that the retro-translocation of mitochondrial proteins and their subsequent degradation in the cytosol is a physiological process that serves to deplete IMS proteins.

The proteome of the IMS is well defined within boundaries of mitochondrial membranes—OM and IM. These boundaries allow for the efficient retention of soluble proteins unless the membrane is permeabilized chemically or by programmed cell death-related events (32). We found that IMS proteins escape from mitochondria, broadening our understanding of the means by which the mitochondrial proteome is maintained. Our recent study identified that IMS proteins constitute a proteasome system in controlling the levels of precursor proteins destined to the IMS (23). The present study indicates that proteins can also relocate from the IMS back to the cytosol and thus become reexposed to the cytosolic quality control machinery. This mechanism can prevent the IMS accumulation of proteins with folding defects and also allow flexible adjustment of the proteome in response to changing needs. Thus, we propose a concept that, in addition to protein import and degradation, the mitochondrial IMS proteome is also maintained by protein retro-translocation to the external cytosolic milieu.

Materials and Methods

Yeast Strains, Growth Conditions, and Biochemical Procedures. The Saccharomyces cerevisiae strains used in this study are listed in Table S1. The growth conditions are described in SI Materials and Methods. The nomenclature for the proteins is according to the Saccharomyces Genome Database. Theoretical mass of proteins is presented in Figs. 1–A to D and 3A, for (g) and b2-Mia40(Δ-mPEG processes). The cell fractionation, protein and mitochondrial isolations, generation of mitoplasts, protease sensitivity assays, and import of radio-labeled precursor proteins into isolated mitochondria were performed according to the standard procedures and are described in SI Materials and Methods.

In Organello Protein Release Assay. Isolated mitochondria were resuspended in the release buffer (250 mM sucrose, 5 mM MgCl2, 80 mM KC, 10 mM Mops/KOH, 5 mM methionine, and 10 mM KH2PO4/HPO4 pH 7.2 for yeast mitochondria; 250 mM sucrose, 5 mM MgGat, 80 mM KOAc, 10 mM sodium succinate, 5 mM methionine, and 20 mM Meps/KOH pH 7.4 for human mitochondria) and incubated at 30 °C or 37 °C for yeast and human samples, respectively, in the presence of the protease inhibitors (P8215, Sigma) for 10 min followed by treatment with or without 50 mM DTT for 30 min or as indicated. Mitochondria were then resolated. Supernatant fractions were precipitated with 10% (v/v) TCA, and mitochondrial pellets were denatured in 2x Laemmli buffer with 100 mM DTT at 65 °C for 15 min. For protein treatment (25 μg/mL), the samples were incubated at 4°C for 15 min and protease inhibitors were omitted.

Cysteine Residue Modification in Intact Mitochondria. Isolated mitochondria were resuspended in the release buffer supplemented with 1.6–2 mM mPEG (5,000 Da) or 2 mM 4′-acetamido-4′-maleimidylstilbene-2′,2′-disulfonic acid (AMS) for 30 min at 30 °C. The corresponding volume of the solvent was used as a mock control. The samples that were used in the subsequent protein release assay were further diluted with the release buffer to ≤1 mM mPEG concentration. Alternatively, the mitochondria were resolated by centrifugation, denatured in reducing Laemmli buffer, and analyzed directly.

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REFERENCES
SI Materials and Methods

Growth Conditions and Generation of Yeast Variants. Yeast were grown in full media YPD/YPS/YPG/YPSGl [% yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) glucose/2% (wt/vol) sucrose/3% (wt/vol) glycerol/2% (wt/vol) galactose, respectively] or in minimal media that contained 0.5% ammonium sulfate and 0.17% yeast nitrogen base, supplemented with suitable nutrients and a carbon source. For proteasome inhibition with MG132 or PS341 (75 μM), the modified minimal medium without ammonium sulfate but with 0.1% proline and 0.003% SDS was used as described previously (1).

The Tom40_C136 and Tom40_C138 variants were prepared on the template of the wild-type Tom40-containing plasmid (pFL39) by removal of native cysteine codons (C165W, C326A, C341S, and C355F) followed by introduction of cysteine codons at new positions (N130C and S138C) (2). The strains expressing Tom40 variants were generated by plasmid shuffling similar to the corresponding wild-type strain (2). For production of β2-Mia40core in wild-type cells that also express native Mia40, yeast were transformed with centromeric plasmids pGB9310 (pFL39-β2-Mia40core; 47) or pPB13.1 (pRS416-β2-Mia40core; 166) for tryptophan or uracil selection, respectively, with the fusion protein placed under the control of native Mia40 promoter and terminator sequences (3). The plasmid with β2-Mia40core was also used as a template for generation of cysteine to serine variants: pGB9477 (pFL39-β2-Mia40core; C15); 48), pPB11.1 (pFL39-β2-Mia40core; C25; 49), pPB12.4 (pFL39-β2-Mia40core; C56; 50), pPB16.2 (pRS416-β2-Mia40core; C56; 168), pGB9479 (pFL39-β2-Mia40core; C56; 51), and pPB17.1 (pRS416-β2-Mia40core; C56; 169). The cysteine residues are numbered as in Böttger et al. (4). For production of FLAG-tagged proteins, yeast were transformed with the pESC-URA plasmid (Ambion) encoding Cox12FLAG (pAG3; 55), Pet191FLAG (pAG1; 53), and Mix17FLAG (pAG2; 54), with the fusion protein under the control of inducible GAL10 promoter. Additionally, based on pAG1, the construct encoding head-to-tail dimer of Pet191 (Pet191_Pet191FLAG) was created (pPB22.1; 170). To induce the GAL10 promoter, addition of 0.5% galactose to the glycerol-containing medium was used. The β2-Cox12FLAG-encoding plasmid (pPB23.1; 212) was prepared based on pPB13.1 with Cox12FLAG recloned from pAG3.

Cell Fractionation and Mitochondrial Procedures. For fractionation, the equivalent of 40 OD600 units of yeast cells grown at respiratory conditions at 37 °C was used. The fractionation procedure was described previously (1). For each fraction, an equivalent of 0.8 OD600 units of cell extract was tested. Yeast mitochondria were isolated using the standard method with differential centrifugation (5) and were stored at -80 °C in the sucrose/Mops (SM) buffer (250 mM sucrose and 10 mM Mops/KOH, pH 7.2). Human mitochondria were isolated from HEK293 cells cultured at 37 °C under 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) FBS and 2 mM l-glutamine. For mitochondria isolation, cells were collected by scraping and homogenized in a Dounce homogenizer in the isolation buffer [300 mM trehalose, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% BSA (wt/vol), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM Hepes/KOH, pH 7.7] (6). Cell debris was removed by centrifugation at 1,000 × g for 10 min. Mitochondria were isolated by centrifugation at 10,000 × g for 15 min, and the mitochondrial pellet was resuspended in the isolation buffer without BSA and directly used without storage. Protein content was quantified using the Bradford assay. To generate mitoplasts, mitochondria were incubated for 30 min on ice in the hypoosmotic buffer indicated in the figure legends (Figs. S2 A and B and S4 A and B). If indicated, samples were split into halves and further incubated with or without 10 μg/mL of proteasome K for 15 min. Proteasome K activity was inhibited by addition of phenylmethylsulfonyl fluoride (PMSF, 2 mM). Mitochondria or mitoplasts were then resolated by centrifugation. Supernatant fractions were precipitated with 10% (wt/vol) TCA.

Import of Radiolabeled Precursor Proteins. The [35S]methionine-labeled precursor proteins were synthesized using the rabbit reticulocyte TNT Quick Coupled Transcription/Translation kit (Promega). The import of radiolabeled precursors into isolated yeast mitochondria was performed at 25 °C in the import buffer [250 mM sucrose, 5 mM MgCl2, 80 mM KCl, 10 mM Mops/KOH, 5 mM methionine, 3% (wt/vol) BSA, 10 mM KH2PO4/KHPO4, pH 7.2] supplemented with 2 mM ATP and 2 mM NADH. Reactions were stopped by cooling on ice and dissipation of the IM potential by addition of 1 μM valinomycin, 20 μM oligomycin, and 8 μM antimycin. To remove nonimported proteins, samples were treated with 25 μg/mL proteinase K for 15 min on ice. Reactions were stopped by addition of 2 mM PMSF. After centrifugation and washing with SM buffer, the samples were denatured in reducing (50 mM DTT) Laemmli buffer and analyzed by SDS/PAGE. Radioactive signal was detected by digital autoradiography.

Miscellaneous. Total cellular protein extracts were prepared by alkaline lysis (7). For immunodetection, enhanced chemiluminescent signals were detected by ImageQuant LAS4010 (GE Healthcare) or X-ray films. For densitometry, ImageQuant TL (GE Healthcare) software was used. In some figures, nonrelevant gel parts were excised digitally. The images were processed using Photoshop CS4 (Adobe).
Fig. S1. (A) Import of [35S]b2(220)-DHFR into WT mitochondria with or without IA (50 mM). Blocking of thiol groups does not affect the presequence-dependent import into mitochondria. (B) Cellular levels of b2-Mia40, core variants in WT, Δyme1, Δatp23, and Δprd1 strains were not changed. Proteins were analyzed by autoradiography (A) or SDS/PAGE and immunodetection (B). i, intermediate; m, mature; p, precursor; WT, wild type.
Fig. S2. 

(A) Protein levels in isolated WT yeast mitochondria or mitoplasts and the corresponding supernatant fractions (release) upon incubation in the release buffer. Mitoplasts were generated by hypotonic swelling in the release buffer without sucrose. Samples were treated with or without proteinase K as indicated. Ccp1, Cyb2, Cyc1, and Mpm1 proteins were efficiently released from mitoplasts. Cox12, Pet191, and Erv1 proteins were partially released from mitoplasts. Cox12, Pet191, Erv1, Cyb2, Mpm1, and Cox11 proteins were sensitive to protease treatment only in mitoplasts, and Tom22 and Tom70 were sensitive to protease treatment in intact mitochondria and mitoplasts. (B) Protein levels in mitoplasts generated from mitochondria of WT yeast expressing b2-Mia40core-C4S and supernatant fractions (release). Mitoplasts were generated by the hypotonic swelling in the SM buffer containing 25 mM sucrose. Samples were treated with or without proteinase K, as indicated. The m-form, but not i-form, of b2-Mia40core-C4S was efficiently released from mitoplasts similar to Pet191, Ccp1, and Cyb2 proteins. Both forms of b2-Mia40core-C4S were sensitive to protease K treatment, similar to Pet191, Erv1, Cyb2, Cox11, Cyc3, Mia40, Tim23, and Tom70 proteins. In A and B, protein extracts were analyzed by SDS/PAGE and immunodetection. WT, wild type.
Fig. S3. Redox state of proteins released from mitochondria upon DTT treatment. (A) Schematic representation of thiol modification by AMS used to determine protein redox state. Only the reduced thiol groups react with AMS, resulting in the increased molecular weight and shift in protein migration on the SDS/PAGE. The modification was applied in the 2× Laemmli buffer. (B) Protein levels and redox states in isolated WT mitochondria and the corresponding supernatants (release) upon treatment with DTT. Pet191 was efficiently released from mitochondria upon DTT treatment, and the released protein was modified with AMS, indicating its reduced state. In contrast, Tim8, Tim10, and Tim13 were largely resistant to DTT treatment, with only a minor fraction found to be reduced and released. Protein extracts were analyzed by SDS/PAGE and immunodetection. Number of cysteine residues present in the mature protein is given in parentheses. Ox, oxidized; red, reduced; WT, wild type.
**Fig. S4.** In organello validation of human mitochondrial protein release susceptibility and protease sensitivity. (A) Protein levels in isolated human HEK293 mitochondria, mitoplasts, and the corresponding supernatant fractions (release) upon incubation in the release buffer. Mitoplasts were generated by hypoosmotic swelling in the release buffer without sucrose. The CYCS protein was released from mitoplasts but not mitochondria. (B) Protein levels in human HEK293 mitochondria, mitoplasts, and supernatants (release). Mitochondria were subjected to the hypoosmotic swelling in 20 mM Hepes buffer, pH 7.4 with 100, 25, or 5 mM sucrose. Samples were treated with or without proteinase K as indicated. In the case of 25 mM and 5 mM sucrose-treated samples (mitoplasts), ALR was found in the release fraction. In the mitoplast samples, ALR, MIC19, and TIMM23 were sensitive to proteinase treatment, whereas TOMM20 was sensitive to proteinase treatment in mitochondria and mitoplasts. In A and B, protein extracts were analyzed by SDS/PAGE and immunodetection.
Fig. S5. Characterization of mitochondria with Tom40 variants. (A) Protein levels in the WT and Δtom5 mitochondrial fractions upon treatment with DTT correspond to the release fractions shown in Fig. 4C. (B) Comparison of protein levels in mitochondria with wild-type Tom40 or Tom40CRFEE and the corresponding supernatants (release) upon treatment with DTT for the indicated time. (C) A schematic representation of S. cerevisiae Tom40 and Tom40C130/C138 with marked positions of cysteine residues at the top. The Tom40C130/C138 model with cysteine residue sulphydryl groups (red) marked with yellow arrowheads in the lumen of the channel at the bottom. The Tom40 homology model generated by Qiu et al. (1) was generated using PyMOL after the side chains of residues 130 and 138 were replaced by cysteines using Coot without changing the main chain (2). (D) Protein levels in mitochondria isolated from strains
expressing wild-type Tom40 or Tom40C130C138. (E) Protein levels in the WT and Tom40C130C138 mitochondrial fractions, pretreated with mPEG followed by the treatment with DTT for the indicated time, correspond to the release fractions shown in Fig. 4E. (F) Analysis of Mix17\text{FLAG} in cellular protein extracts and isolated mitochondria. (G) Protein levels in the mitochondria with Tom40C130C138 and Mix17\text{FLAG}, with or without pretreatment with mPEG, upon treatment with DTT for the indicated time, correspond to release fractions shown in Fig. 4F. In A, B, and D–G, protein extracts were analyzed by SDS/PAGE and immunodetection. WT, wild type; *, full-size Mix17\text{FLAG}; **, Mix17\text{FLAG} degradation product.

Fig. S6. The respiration-to-fermentation shift induces IMS protein susceptibility to proteasomal clearance. (A) Cellular protein levels upon shift from respiration to fermentation with or without proteasome inhibitor. Final concentration of the proteasome inhibitors MG132 and PS341 was 75 μM. Experimental scheme is provided at the top. (B) Cellular levels of Cox12\_FLAG and Pet191\_FLAG upon expression induction pulse (T0), after 2 h chase without induction (T1) and upon shift from respiration to fermentation with or without MG132 (T2). Experimental scheme is provided at the top. (C) Growth test of WT and Δcox12 cells transformed with the b2-Cox12\_FLAG–encoding plasmid or empty vector. Serial 10-fold dilutions of yeast cells were analyzed on minimal medium with carbon source as indicated. In A and B, protein extracts were analyzed by SDS/PAGE and immunodetection. WT, wild type.
**Table S1. S. cerevisiae strains used in this study**

<table>
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<tr>
<th>Strain (laboratory ID no.)</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>YPH499 (524)</td>
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<td>Erv1-2 (235)</td>
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<td>Tom40 (772)</td>
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</tr>
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<td>WT for tom40_{C130C138} and tom40_{CFREE} (771)</td>
<td>MATa, ade2-101, his3-D200, leu2-α1, ura3-52, trp1-Δ63, lys2-801, tom40::ADE2 [pFL39-TOM40-C165W/C326A/C341S/C355F]</td>
<td>(4)</td>
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<td>tom40_{C130C138} (769)</td>
<td>MATa, ade2-101, his3-D200, leu2-α1, ura3-52, trp1-Δ63, lys2-801, tom40::ADE2 [pFL39-TOM40-N130C/S138C/C165W/C326A/C341S/C355F]</td>
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