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The conserved AAA-ATPase Msp1 confers organelle specificity to tail-anchored proteins

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The accuracy of tail-anchored (TA) protein targeting to the endoplasmic reticulum (ER) depends on the Guided Entry of Tail-Anchored (Get) protein targeting machinery. The fate of TA proteins that become inappropriately inserted into other organelles, such as mitochondria, is unknown. Here, we identify Msp1, a conserved, membrane-anchored AAA-ATPase (ATPase associated with a variety of cellular activities) that localizes to mitochondria and peroxisomes, as a critical factor in a quality control pathway that senses and degrades TA proteins mistargeted to the outer mitochondrial membrane (OMM). Pex15 is normally targeted by the Get pathway to the ER, from where it travels to peroxisomes. Loss of Msp1 or loss of the Get pathway results in the redistribution of Pex15 to mitochondria. Cells lacking both a functional Get pathway and Msp1 accumulate increased amounts of Pex15 on the OMM and display severely dysfunctional mitochondrial morphology. In addition, Msp1 binds and promotes the turnover of a Pex15 mutant that is misdirected to the OMM. Our data suggest that Msp1 functions in local organelle surveillance by extracting mistargeted proteins, ensuring the fidelity of organelle specific localization of TA proteins.

Correct targeting of proteins to appropriate subcellular compartments is critical for cell organization and physiology. In addition to the well-established signal sequence-directed protein targeting pathways to subcellular organelles (1), some classes of transmembrane proteins use more recently discovered targeting routes. One class comprises tail-anchored (TA) proteins that contain a hydrophobic stretch of amino acids at their extreme C terminus. TA proteins are posttranslationally targeted to the endoplasmic reticulum (ER), mitochondria, and peroxisomes. Many specify organelle identity or are otherwise essential for proper organelle function, including SNAREs, ubiquitin ligases, and organelle division machinery. An ATP-dependent protein targeting pathway termed the Guided Entry of Tail-Anchored (Get) pathway in Saccharomyces cerevisiae (2) and the Asna1/TRC40 pathway in higher eukaryotes (3) catalyzes insertion of TA proteins into the ER (Fig. 1A, WT). In S. cerevisiae, the Get pathway is composed of the pretargeting factors Get4, Get5, and Sgt2 which deliver newly synthesized TA proteins from ribosomes to the cytosolic targeting ATPase Get3 (4). TA proteins loaded onto Get3 are then handed off to the ER-resident Get1/Get2 receptor complex, which stimulates the release and insertion of the substrate into the ER membrane (2, 5, 6). The Get pathway also inserts the peroxisomal TA protein factor Pex15 into the ER, from where it is trafficked to peroxisomes as they mature (2, 7).

A dedicated targeting pathway for outer mitochondrial membrane (OMM) TA proteins has not been identified. TA protein targeting to mitochondria is thought to rely on a bipartite topogenic signal composed of a weakly hydrophobic transmembrane segment C-terminally flanked by a small (three to four amino acid) stretch of positively charged residues (8). How these features are recognized and distinguished from those of ER-directed TA proteins remains unclear. In S. cerevisiae, the targeting of OMM-localized TA proteins has also been proposed to be guided by the lipid composition of the membrane, in which a low ergosterol content may facilitate the spontaneous insertion of OMM-directed TA proteins (9–11).

The fidelity of partitioning TA proteins between the mitochondria, peroxisomes, and ER is of critical importance to the cell, and as such, ER TA protein targeting is subject to quality control systems that couple failure of membrane integration with substrate ubiquitylation and proteasomal degradation (12). However, the fate of ER TA proteins that escape this preemptive quality control pathway and are mistargeted to the inappropriate organelles is unclear. The observations that disruption of the Get pathway leads to the mistargeting of a subset of ER-directed TA proteins to mitochondria (Fig. 1A, get2Δ and ref. 2) suggests that TA protein targeting to the OMM may be a default targeting pathway that bypasses the quality control systems in place for ER-directed clients. Such a model implies that the OMM would be vulnerable to accumulation of mistargeted proteins, which could become detrimental to mitochondrial function. Thus, we reasoned that a pathway may exist that surveys the OMM, senses mistargeted TA proteins, and promotes their extraction and degradation.

A potential candidate for surveillance of the OMM for mistargeted TA proteins would have two features. First, it would have biochemical characteristics associated with central members of previously defined protein quality control systems, such as Cdc48, an AAA-ATPase that has an essential role in ER-associated protein degradation. Second, it would be localized to the OMM. One such candidate is the highly conserved AAA-ATPase Msp1 (13). Msp1 is an OMM protein containing an N-terminal transmembrane domain followed by a C-terminal cytoplasmic ATPase domain (Fig. 1B and ref. 13). High-throughput studies suggest that Msp1 oligomerizes, likely

Significance

Membrane protein targeting to the endoplasmic reticulum, peroxisomes, and mitochondria requires specialized machinery to ensure the appropriate localization of proteins, which is important for defining organelle identity. Additional specificity is provided by sensing and degrading proteins that are mistargeted to an inappropriate compartment. One class of membrane proteins, which contain a hydrophobic segment at their extreme C terminus (called tail-anchored proteins), are prone to be mistargeted; however, how cells cope with this burden is unknown. In this work, we identify a conserved ATPase of the outer mitochondrial membrane, Msp1, which we propose functions as an extraction engine to remove and initiate degradation of an inappropriate targeted tail-anchored protein. In this way, Msp1 serves to enhance the fidelity of protein localization.

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into a hexameric structure akin to other AAA-ATPases (14). Unlike Cdc48, which contains two ATPase domains that form a double-ring structure (15, 16), Msp1 contains a single ATPase domain composed of canonical Walker A (P-loop) and B (DExx) motifs (Fig. 1B). The presence of a “second region of homology” motif distinguishes Msp1 as a classical AAA-ATPase from the broader AAA\(^+\)-ATPase superfamily members lacking this domain (17).

In this study, we show that Msp1 is localized to the OMM and to peroxisomes and that it acts to prevent the accumulation of the Get-client Pex15 on mitochondria. Our analyses indicate that Msp1 alleviates mitochondrial-specific stress associated with mistargeted TA proteins by promoting their extraction from the OMM.

**Results**

**Msp1 Is Localized to Mitochondria and Peroxisomes.** To localize Msp1, we tagged its gene at its C terminus with green fluorescent protein (GFP) and analyzed the subcellular distribution of the resulting fusion protein by fluorescence microscopy in live cells. In agreement with previous studies (13), Msp1 distributed to mitochondria; however, we also observed numerous extra-mitochondrial foci that did not colocalize with a red-fluorescent mitochondrial marker (mt-dsRED). Arrows indicate extramitochondrial Msp1 foci. Lower, Z-projections of cells expressing chromosomally tagged Msp1-GFP and Pex3-Redstar2. (Scale bar, 4 \(\mu\)m, applies to all panels.)

Fig. 1. Msp1 is an N-terminally anchored AAA-ATPase distributed in the mitochondrial outer membrane and peroxisomes. (A) WT: A schematic illustrates the Get-dependent targeting of ER-destined TA proteins and the incorporation of specific TA proteins into peroxisomes. Mistargeted TA proteins are sensed and degraded. \(\text{get}^\Delta\): In the absence of the dedicated ER-targeting machinery, a subset of ER-destined TA proteins accumulate in the mitochondria, where the degradation machinery becomes important for preventing toxic levels of mistargeted TA proteins from accumulating. \(\text{msp}1^\Delta\): In the absence of Msp1, mitochondria accumulate mistargeted ER TA proteins. (B) Hydropathy of Msp1 plotted as a function of amino acid calculated from TMPred (www.ch.embnet.org). A schematic of Msp1 showing predicted structural features is aligned with the hydrophy plot. The Walker A/B and second region of homology motifs are part of the AAA-ATPase conserved module. (C, Upper) Z-projections of cells expressing chromosomally tagged Msp1-GFP and episomally expressed mt-dsRED. Arrows indicate extramitochondrial Msp1 foci. Lower, Z-projections of cells expressing chromosomally tagged Msp1-GFP and Pex3-Redstar2. (Scale bar, 4 \(\mu\)m, applies to all panels.)

msp\(1^\Delta\): In the absence of Msp1, mitochondria accumulate mistargeted ER TA proteins.
with get2 and get3 deletion mutants (18, 19). To expand on these
data, we compared the growth of wild-type, msp1Δ, get1Δ, get2Δ,
get3Δ, and double mutants of msp1Δ and each Get pathway
member. We observed strong synthetic defects between msp1Δ
and get1Δ, get2Δ, and get3Δ (Fig. 2A, YPD) and less pronounced
interactions between msp1Δ and get4Δ and get5Δ (get4 and get5
mutants were not pursued further in this work).

The growth phenotypes of the msp1Δ get1Δ, msp1Δ get2Δ, and
msp1Δ, get3Δ double-mutant cells were reminiscent of strains
defective in mitochondrial respiration. We tested this possibility
by analyzing the growth of each double mutant on a non-
fermentable carbon source (glycerol), which requires mitochon-
drial respiration. Consistent with this notion, we observed an
enhanced growth defect of the double mutants compared with
either single mutant (Fig. 2A, YPEG). Unexpectedly, although
msp1Δ mutant strains did not display a detectable growth defect
in these conditions, get1Δ, get2Δ, and get3Δ mutant strains
showed substantially impaired growth. These results indicate
that a functional Get pathway is important for mitochondrial
physiology, perhaps, in part, by preventing mistrargeting of normally
ER-targeted proteins to the OMM.

Maintenance of normal mitochondrial morphology commonly
 correlates with accurate inheritance of mtDNA and the ability
of cells to grow on nonfermentable carbon sources. We therefore
compared the mitochondrial morphology of wild-type, single-
mutant, and double-mutant cells (Fig. 2B, images). We quanti-
ﬁed these morphology defects by binning cells into one of three
groups: class 1, tubular, characterized by the elongated, tubular
morphology of mitochondria in wild-type cells; class 2, clumped/
tubular, characterized as an intermediate morphology in which
mitochondria have partially collapsed into clumps but still have
mostly wild-type tubules; and class 3, clumped, characterized by
collapsed mitochondria with few emanating tubules (Fig. 2B,
graph). By this criterion, msp1Δ mutant cells were indistin-
guishable from wild-type cells, showing no overt defects in mito-
chondrial morphology. In contrast, get1Δ, get2Δ, and get3Δ
mutant cells showed a distinct morphology defect, displaying
mostly class 2 cells. Strikingly, double-mutant cells showed strong
synergistic defects displaying mostly class 3 cells. These data suggest
that Msp1 acts to alleviate a mitochondria-specific defect imparted
by compromised Get-dependent TA protein targeting.

The Loss of Msp1 Function Leads to the Mislocalization of Pex15. As
mentioned earlier, a possible explanation for these results is that
loss of Msp1 leads to the accumulation of off-pathway targeted
Get client proteins to the OMM. To test this notion, we examined
the localization of GFP-tagged ER- and peroxisomal-localized TA
proteins in msp1Δ mutant cells. Screening a collection of 15 pro-
tein constructs (listed in Experimental Procedures), we found a
single protein, GFP-Pex15, that in msp1Δ mutant cells displayed a
dramatically altered localization compared with wild-type cells:
Whereas GFP-Pex15 localized exclusively to peroxisomes in
wild-type cells, it also accumulated on mitochondria in msp1Δ
mutant cells (Fig. 3A).

GFP-Pex15 was also mistrargeted to the OMM in cells in which
the Get pathway was compromised (Fig. 3B, Upper), in agreement
with previous observations (1). To test for synergistic effects with
Msp1 disruption, we examined GFP-Pex15 in get3Δ msp1Δ
double-mutant cells. We found that the accumulation of
GFP-Pex15 on the clumped mitochondria was strongly exacer-
bated (Fig. 3B, Lower). We obtained indistinguishable results in
get1Δ msp1Δ and get2Δ msp1Δ double-mutant cells (Fig. S1).
Taken together, these data support the notion that Msp1 acts
downstream of the Get pathway to clear mistargeted Pex15
from mitochondria.

Overexpression of Msp1 Clears Mistargeted Pex15 from the OMM. If
the normal role of Msp1 is to clear mistrargeted TA proteins from
mitochondria, then the accumulation of mistrargeted GFP-Pex15
in get3Δ mutant cells (Fig. 3B) suggests that Msp1 activity in
these cells is insufﬁcient when the normal targeting machinery
(i.e., the Get pathway) is compromised. We tested this notion
directly by overexpressing Msp1 from the GAL1 promoter in
wild-type and get3Δ mutant cells and examining the localization
of GFP-Pex15. In wild-type cells, overexpression of Msp1 did not
alter the distribution of GFP-Pex15, which was exclusively found
on peroxisomes. In contrast, in get3Δ mutant cells, Msp1 over-
expression caused a striking relocation of GFP-Pex15 from

Fig. 2. Msp1 displays synthetic genetic interactions with Get pathway members. (A) Serial dilutions of indicated strains spotted on either fermentable
dextrose media (YPD) or nonfermentable glycerol media (YPEG). (B) Z-projections of indicated strains episomally expressing mt-dsRED grown in selective
dextrose media. (Scale bar, 4 μm, applies to all panels.) The bar graph shows distribution of mitochondrial morphologies in wild-type (n = 29), msp1Δ (n = 38),
get1Δ (n = 35), get2Δ (n = 39), get3Δ (n = 44), msp1Δ get1Δ (n = 34), msp1Δ get2Δ (n = 31), and msp1Δ get3Δ (n = 34) cells.
the OMM into the cytoplasm while leaving GFP-Pex15 localized to peroxisomes unaffected (Fig. 3C).

**Mistargeted Pex15 Is Cleared More Slowly from Mitochondria in msp1Δ Cells.** We next asked whether Msp1 acts on mistargeted Pex15 in the presence of a functional Get pathway. To this end, we altered the Pex15 TA-targeting signal to misdirect the protein. Pex15 contains an extended 34-amino acid stretch beyond the transmembrane anchor, which we truncated just beyond a short stretch of basic amino acids (KKYK), creating GFP-Pex15-Δ30. We reasoned that this truncation would create a protein structurally resembling a mitochondrial-targeted TA protein (8). Indeed, when we examined GFP-Pex15-Δ30 localization in wild-type and msp1Δ mutant cells, we found that

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**Fig. 3.** Msp1 controls the steady-state distribution of Pex15. (A and B) Z-projections of indicated strains episomally expressing matrix-targeted TagBFP (mt-TagBFP) and GFP-Pex15. (Scale bar, 4 μm.) (C) Z-projections of indicated strains episomally expressing GFP-Pex15 grown in inducing conditions (galactose) for 4 h to overexpress Msp1. (Scale bar, 4 μm.)
the truncation caused a marked relocation to mitochondria (Fig. 4A).

We next used GFP-Pex15Δ30 to probe the clearance of the mutant protein from the mitochondrial membrane. In wild-type cells expressing GFP-Pex15Δ30 under the control of the regulatable GAL1 promoter, shifting from galactose (inducing) media to dextrose (repressing) media led to the rapid degradation of GFP-Pex15Δ30 (t1/2 = 34 ± 7 min; Fig. 4B). In contrast, in msp1Δ mutant cells, GFP-Pex15Δ30 was stabilized, increasing its t1/2 ~threefold (t1/2 = 87 ± 5 min; Fig. 4B). The carbon source switch did not change the doubling times of wild-type or msp1Δ mutant cells (wild-type, t1/2 = 117 ± 11 min; msp1Δ, t1/2 = 116 ± 4 min). Taken together with the observation that the levels of GFP-Pex15Δ30 in wild-type cells at t = 0 were markedly lower (~13-fold) than those in msp1Δ mutant cells (Fig. 4B, Left), these results show that cells containing Msp1 constitutively degrade GFP-Pex15Δ30.

Msp1 Interacts with Mistargeted Pex15. The stimulation of Pex15-Δ30 degradation in cells containing Msp1 suggested that Msp1 functionally interacts with mistargeted Pex15 to promote its degradation. To test for a physical interaction, we C-terminally tagged Msp1 with 3xFLAG to use in pull-down experiments. We also generated a tagged variant of Msp1 mutated in the Walker B motif (Msp1-E193Q) that, by analogy to other AAA-ATPases, is predicted to impair ATP hydrolysis and trap interactions with putative substrates (20). Expression of Msp1-3xFLAG in msp1Δ cells stimulated the turnover of GFP-Pex15-Δ30 (Fig. 4C, compare lanes 1 and 4), suggesting the 3xFLAG tag does not impair Msp1 function. In contrast, Msp1-E193Q-3xFLAG did not stimulate the turnover of GFP-Pex15-Δ30 (compare lanes 1 and 9), demonstrating that nucleotide hydrolysis is important for Msp1 function. Immunoprecipitation of Msp1-3xFLAG coprecipitated Pex15-Δ30 (Fig. 4C, lanes 6–8), indicating that Msp1 physically interacts with GFP-Pex15-Δ30. The coprecipitation of GFP-Pex15-Δ30 was stimulated ~fivefold (normalized for variable levels of GFP-Pex15-Δ30 in the input) by expressing the substrate trap mutant Msp1-E193Q (compare lanes 6–8 with lanes 11–13). These data suggest that Msp1 binds to mistargeted mitochondrial TA proteins in an ATPase-modulated manner to stimulate their degradation.

Discussion
Proper intracellular protein localization is essential to maintain the compartmentalization of eukaryotic cells. This organization is achieved by the interplay of protein-targeting pathways that recognize features of newly synthesized proteins to bring them to the correct locale and quality control pathways that extract and degrade mislocalized proteins. We here provide evidence that the conserved, integral membrane AAA-ATPase Msp1 is localized to both mitochondria and peroxisomes. We show that on mitochondria, Msp1 plays a role in a previously undescribed pathway that promotes extraction and degradation of the peroxisomal protein Pex15 when it is mistargeted to the OMM (Fig. 1A, msp1Δ). The role of Msp1 in assuring the correct, peroxisome-specific localization of Pex15 is supported by three lines of evidence. First, loss of Msp1 function leads to the accumulation of Pex15 on the OMM. This result indicates that in normal, wild-type cells, a portion of Pex15 molecules are constitutively mistargeted and that the defect is then corrected by Msp1. Second, in cells where mislocalization of Pex15 to the OMM was exacerbated by compromising the Get pathway (which normally ensures proper Pex15 targeting to the peroxisomes via the ER), Msp1 stimulates the extraction of mistargeted Pex15 in the OMM. Third, Msp1 physically interacts with and accelerates the nucleotide-hydrolysis-dependent turnover of a Pex15 mutant that is inappropriately targeted to the OMM. Although our analyses are focused on a single TA protein, Pex15, Msp1 may similarly act on other mistargeted membrane proteins. Moreover, the dual localization of Msp1 to both the OMM and peroxisomes suggests, by extension, that reciprocally, Msp1 may play a similar role in both organelles.

Precedence for a role of AAA-ATPases in membrane protein extraction is provided by Cdc48, which functions in ER-associated protein degradation and in mitochondrial-associated degradation during conditions of elevated oxidative stress (21). Cdc48 is thought to use the energy of ATP hydrolysis to extract and unfold proteins, readying them for subsequent delivery and degradation by the proteasome. In contrast to Cdc48, Msp1 is predicted to contain only a single hexameric ATPase ring and a bona fide transmembrane segment, which must firmly anchor and orient the oligomeric assembly in the OMM and peroxisomal membrane. Thus, although the use of AAA-ATPases in membrane protein extraction may highlight a common principle of organelle protein surveillance systems, the mechanisms by which Cdc48 and Msp1 perform their respective tasks are likely to be different. In this light, it will be particularly intriguing to assess how Msp1 can distinguish proteins that are in the correct membrane from those that have been mistargeted. A distinguishing feature could be surface determinants that become buried only when the TA protein interacts with partners found exclusively in its appropriate locale. In the absence of such binding partners, these determinants would remain exposed and may be recognized by Msp1 or some cofactor or cofactors yet to be identified, marking the protein for extraction and degradation.

Get pathway mutants, but not msp1Δ mutants, display a severely compromised mitochondrial morphology and function (ref. 22 and this study). Thus, Msp1 clears basal levels of mistargeted ER clients during the course of normal growth and becomes limiting when the Get pathway is compromised. Taken together, these data suggest that in wild-type cells, the basal levels of TA protein mistargeting during normal growth is low but may increase when cells are exposed to environmental stresses or nutrient limitation, thus necessitating Msp1 function.

The high degree of homology of Msp1 in eukaryotes suggests that aspects of the TA protein mislocalization sensing pathway described here in S. cerevisiae are conserved in higher eukaryotes; however, the role of metazoan Msp1 homologs, ATAD1/Thorase, for this function has yet to be explored experimentally. In support of a similar role in both organelles, the role of ATAD1 in targeting ER clients during the course of normal growth and becomes limiting when the Get pathway is compromised. Taken together, these data suggest that in wild-type cells, the basal levels of TA protein mistargeting during normal growth is low but may increase when cells are exposed to environmental stresses or nutrient limitation, thus necessitating Msp1 function.

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Experimental Procedures
Yeast Strains and Plasmids. Yeast strains used in this study are listed in Table S1. Strains with chromosomal integrations were constructed by homologous recombination of PCR products (22). GFP-Pex15 was made by PCR amplifying GFP5657T with flanking SpeI and HindIII sites, Pex15 with flanking HindIII and XhoI sites, and cloning both fragments into p416CYC, yielding p416CYC-GFP-Pex15. N-terminal GFP fusions were made by PCR amplifying TagFP containing the mitochondrial Su9 presence and TagFP was subcloned into p413GPD, yielding p413GPD-GFP-Pex15-Δ30. Msp1-3xFLAG was made by PCR amplifying TagFP with flanking BamHI and HindIII sites, and cloning Msp1 into p413GPD-Δ30. Msp1-3xFLAG was made by PCR amplifying TagFP with flanking BamHI and HindIII sites, and cloning Msp1 into p413GPD-Δ30. Msp1-3xFLAG was made by PCR amplifying TagFP with flanking BamHI and HindIII sites, and cloning Msp1 into p413GPD-Δ30. Msp1-3xFLAG was made by PCR amplifying TagFP with flanking BamHI and HindIII sites, and cloning Msp1 into p413GPD-Δ30. Msp1-3xFLAG was made by PCR amplifying TagFP with flanking BamHI and HindIII sites, and cloning Msp1 into p413GPD-Δ30.
Msp1-E193Q-3xFLAG was generated using a single oligonucleotide mutagenesis approach, as described (29).

**Cell Imaging.** Strains were cultivated in SD –Trp lacking the appropriate nutrient for selection of episomal construct at 30 °C at early to midlog phase (OD<sub>600</sub> ~0.3–0.5), immobilized on coverslips coated with 0.1 mg/mL Con A (Sigma), and imaged using either a Zeiss Axiovert 200M microscope equipped with a 100× 1.3 NA objective and a CCD camera (Orca-ER; Hamamatsu Photonics; Figs. 1C, Lower, and 2B, 3C, and 4A) or a Nikon Eclipse Ti equipped with a 100× 1.25 NA objective (CSU-X1; Yokogawa), EMCCD camera (Xon3 897; Andor), and a 100× 1.49 NA objective (Figs. 1C, Upper, and 3A and B). Images were acquired with μManager software (30) and processed with ImageJ 1.46r (http://rsb.info.nih.gov/ij). Mitochondrial morphology was classified from maximum-intensity projections, using the following criteria. Cells with tubular (class 1) mitochondria were defined as having no mitochondrial structures more than 600 nm in width, which corresponds to 2 times the average width of a mitochondrial tubule in the majority of wild-type cells under our imaging regime. Cells with clumped/tubular (class 2) mitochondria were defined as having any part of the mitochondrial mass with a diameter more than 600 nm but with more than half of the mitochondrial mass with a width of no more than 600 nm. Cells with clumped (class 3) mitochondria were defined as having any part of the mitochondrial mass with a diameter more than 600 nm but with less than half of the mitochondrial mass with a diameter no more than 600 nm.

**Galactose Shutoff Experiment.** Cells were grown in SD medium for 8 h to the postdiauxic shift (OD<sub>600</sub> ~1.0), harvested by centrifugation, washed with water, and resuspended in SGaL [2% (vol/vol) galactose]. Cells were induced for 9 h, harvested, washed once with water, and resuspended in SD –Leu to OD<sub>600</sub> ~0.2. Cells were harvested every hour and processed to cell extract as per ref. 31, with the following modifications: 1.5× 10<sup>6</sup> cells were processed per time point, and cells were boiled in 1% SDS and 100 mM Tris·HCl at pH 7.5 (at 25 °C) for 5 min and then flash-frozen. The cell lysate was rapidly thawed, diluted 10 times with a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris·HCl at pH 7.5, and left on ice for 10 min. Cell debris was spun down at 10,000 × g for 10 min at 4 °C. Lysate from 4× 10<sup>6</sup> cells was loaded on an Any kD Mini-PROTEAN TGX gel (Bio-Rad). GFP-PerX5·s30 was detected with rabbit anti-GFP antibodies (Invitrogen) and goat anti-rabbit IRDye 680, using an infrared imaging system (Olympus; Li-COR Biosciences). Quantification of IR signals was performed with ImageQuant 5.2 (GE Healthcare).

**Immunoprecipitations.** Cells were grown in SD –Leu –Ura to log phase, harvested by centrifugation, washed with water, and resuspended in SGal –Leu –Ura media to OD<sub>600</sub> 0.5 and grown for 9 h. One hundred OD<sub>600</sub> units of cells were harvested by centrifugation, washed once with water, and resuspended in ice-cold IP Buffer [50 mM Hepes KCl at pH 7.5, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 200 mM sorbitol, 1 mM NaF, 0.1% digitonin, 10 μL (0.5×) protease inhibitors, EDTA-free (Roche)]. Cells were snap frozen in liquid nitrogen and rapidly thawed in water twice. Glass beads ~0.5 mm in size were added, and the cells were vortexed at 4 °C for 10 min. Cell lysate was removed from the beads, digitonin was added to 1%, and the cell lysate was solubilized at 4 °C for 45 min. Lysate was clarified by centrifugation at 15,000 × g for 15 min at 4 °C, and the supernatant was diluted with IP Buffer to 1× and incubated for 1 h with 5 μL of anti-GFP magnetic beads at 4 °C. The beads were washed 4 times with IP Buffer and then boiled in 100 μL Laemmli sample buffer for 5 min to release bound material. Of the bound material, 1 μL (1×), 5 μL (5×), and 10 μL (10×) was loaded on the gel in Fig. 4C.

**Note Added in Proof.** In agreement with our conclusions, Chen et al. (32) independently demonstrated that Msp1 and ATAD1/thorase promote the degradation of mislocalized TA proteins at the OMM.

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