

*Annual Review of Cell and Developmental Biology*  
**Msp1/ATAD1 in Protein  
 Quality Control and  
 Regulation of Synaptic  
 Activities**

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**Abstract**

Mitochondrial function depends on the efficient import of proteins synthesized in the cytosol. When cells experience stress, the efficiency and faithfulness of the mitochondrial protein import machinery are compromised, leading to homeostatic imbalances and damage to the organelle. Yeast Msp1 (mitochondrial sorting of proteins 1) and mammalian ATAD1 (ATPase family AAA domain-containing 1) are orthologous AAA proteins that, fueled by ATP hydrolysis, recognize and extract mislocalized membrane proteins from the outer mitochondrial membrane. Msp1 also extracts proteins that have become stuck in the import channel. The extracted proteins are targeted for proteasome-dependent degradation or, in the case of mistargeted tail-anchored proteins, are given another chance to be routed correctly. In addition, ATAD1 is implicated in the regulation of synaptic plasticity, mediating the release of neurotransmitter receptors from postsynaptic scaffolds to allow their trafficking. Here we discuss how structural and functional specialization imparts the unique properties that allow Msp1/ATAD1 ATPases to fulfill these diverse functions and also highlight outstanding questions in the field.

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### 1. AN OVERVIEW OF Msp1-MEDIATED MITOCHONDRIAL PROTEIN QUALITY CONTROL

Mitochondria are complex organelles that serve numerous functions, including ATP synthesis, iron–sulfur cluster assembly, metabolism, proteostasis, and apoptosis (Pfanner et al. 2019). They contain over a thousand different proteins, localized in four different compartments: the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM), the intermembrane space (IMS), and the matrix space. The vast majority of these proteins are encoded in the nucleus and imported into the organelle from the cytosol (Wiedemann & Pfanner 2017). To ensure their proper function, mitochondria employ multiple mechanisms for proteins to be recognized, imported, and then sorted into the correct compartment. However, all protein targeting reactions are prone to mistakes. To prevent the mistargeted proteins from accumulating in the organelle, mitochondria must recognize and reject noncognate proteins. Recent studies have revealed that the yeast AAA (ATPase associated with diverse cellular activities) protein Msp1 (mitochondrial sorting of proteins 1) functions in mitochondria to ensure proper protein import and to correct such targeting mistakes, establishing it as a key factor in mitochondrial protein quality control. In addition, Msp1’s mammalian homolog ATAD1 (ATPase family AAA domain–containing 1) has been implicated in having a crucial role in the regulation of synaptic activities in neurons. In this review, we synthesize the current knowledge of the Msp1-mediated mitochondrial protein quality control pathways and highlight the outstanding questions in the field.

#### 1.1. The Extraction of Mislocalized Tail-Anchored Proteins from the Outer Mitochondrial Membrane

Membrane proteins constitute about one-third of the cell’s proteome (Wallin & Heijne 2008). Upon synthesis, cells must precisely route each membrane protein to the correct intracellular locale to ensure its proper function. The successful delivery of a membrane protein to its appropriate organelle requires matching its signal sequence with the targeting machinery that recognizes it.

For example, endoplasmic reticulum (ER)-targeted proteins possess N-terminal signal sequences that are recognized by the signal-recognition particle (SRP) upon emerging from the ribosome. They are then delivered to the ER membrane, where they are cotranslationally inserted into the bilayer by the ER-resident Sec61 translocon (Akopian et al. 2013). The signal sequence is usually proteolytically removed. The cotranslational targeting mechanism ensures the specificity of the targeting reaction. By contrast, tail-anchored (TA) proteins cannot use this mechanism, because the targeting information in their C-terminal membrane anchors only becomes exposed outside the ribosome after the termination of protein synthesis.

TA proteins constitute about 3–5% of the eukaryotic membrane proteome (Beilharz et al. 2003, Kalbfleisch et al. 2007, Kriechbaumer et al. 2009). They are integral membrane proteins that are embedded in the membrane exclusively via a hydrophobic stretch in the C-terminus. TA proteins function in important physiological processes such as apoptosis (Bcl-2 family proteins), protein import [translocase of the outer membrane 20 (Tom20)], vesicle targeting and fusion [soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins], and organelle biogenesis [peroxisomal membrane protein 15 (Pex15)]. Their organelle-specific information is encoded at the extreme C-terminus; hence, these proteins are posttranslationally targeted to organellar membranes (Borgese & Fasana 2011). The hydrophobic sequences serve as both targeting signals and membrane anchors.

Dedicated cellular machinery delivers TA proteins to their cognate organellar membranes. For TA proteins that travel to or through the ER, the Get proteins [in yeast; transmembrane domain recognition complex (TRC) proteins in humans] deliver them to the ER membrane. In particular, Get3 loads the newly synthesized TA proteins in an ATP-dependent manner (Schuldiner et al. 2008) and hands them over to the ER-resident receptors Get1 and Get2 that are thought to form a membrane insertase complex (Denic 2012). After insertion, they stay in the ER, travel to peroxisomes, or populate the membranes of organelles in the secretory pathway such as the Golgi apparatus and the plasma membrane. By contrast, the insertion of TA proteins into the mitochondria is Get independent. For a long time, no mitochondrial insertase was discovered for TA proteins and the process was thought to occur spontaneously (Chio et al. 2017); however, this notion was challenged by a recent study (Doan et al. 2020) which reported that the mitochondrial import complex could assist the import of tail-anchored proteins.

Partitioning between the ER- and the mitochondria-targeted TA proteins is determined by a combination of several properties encoded at the extreme C-terminus, including the transmembrane domain (TMD) and the C-terminal element (CTE) that follow it. On average, the ER TA proteins have longer transmembrane regions that are more hydrophobic and have a higher propensity to form an alpha helix, whereas the OMM TA proteins have TMDs that are shorter and less hydrophobic with a lower helical propensity (Beilharz et al. 2003, Lee et al. 2014, Rao et al. 2016). In addition, some OMM TA proteins are enriched in positively charged amino acids in the CTE (Chio et al. 2017).

Besides these subtle differences, the biophysical properties of the ER- and the mitochondria-targeted TA proteins are rather similar, which causes their mistargeting. Mistargeting is exaggerated in cells with malfunctioning Get machinery (Okreglak & Walter 2014) but also occurs at a baseline level in normal cells. This necessitates a proofreading mechanism especially on the mitochondria, where TA protein insertion could occur spontaneously. This function is carried out by Msp1.

The first hint of the function of Msp1 in TA protein quality control came from its strong genetic interaction with the Get proteins. Two independent studies (Chen et al. 2014, Okreglak & Walter 2014) showed that while deleting Msp1 or a gene from the Get pathway alone does not result in a significant growth defect, deleting both causes a synthetic growth defect in yeast,

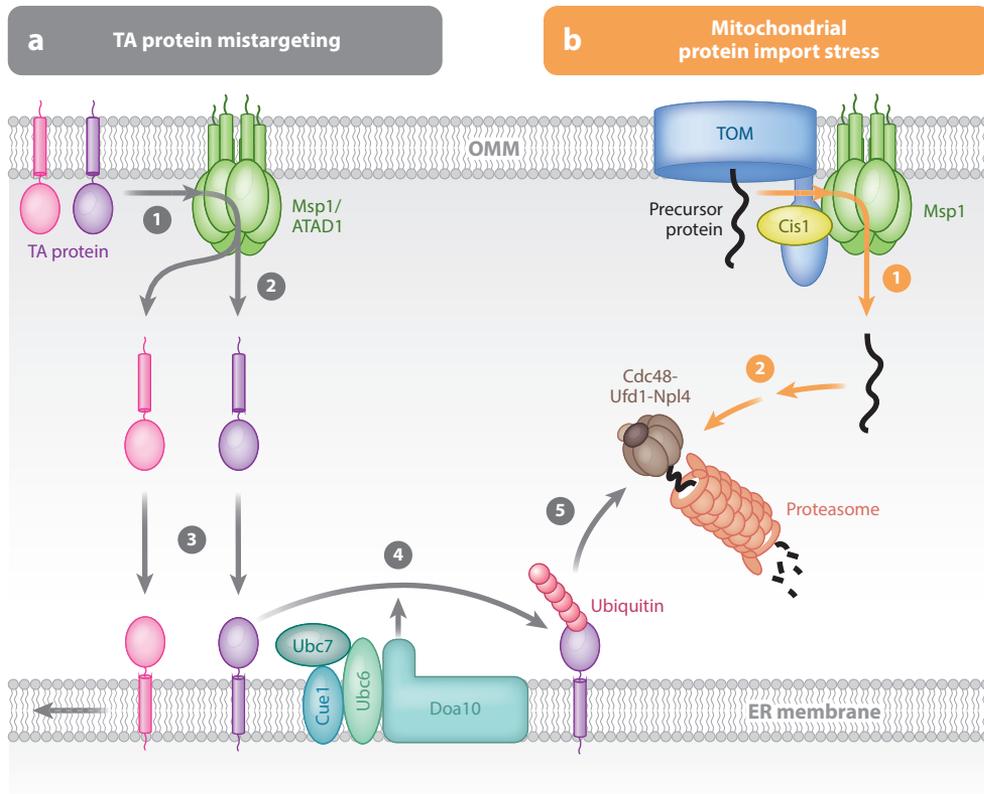
indicating that the Get proteins and Msp1 function in parallel, genetically linked pathways. Imaging of the peroxisomal TA protein Pex15 showed that in *get3* $\Delta$  cells a small fraction of Pex15 is mislocalized to mitochondria (Chen et al. 2014, Okreglak & Walter 2014). The overexpression of Msp1 in these cells leads to the depletion of mislocalized Pex15 (Chen et al. 2014). By contrast, the deletion of Msp1 greatly exacerbates the mislocalization effect (Okreglak & Walter 2014), indicating that Msp1 counters the effect of TA protein mislocalization caused by *get3* deletion. In vitro reconstitution experiments showed that Msp1 achieves this effect by extracting the TA proteins from the OMM in an ATP-dependent manner (Wohlever et al. 2017). The same function has been shown in the human ATAD1 in mammalian cells (Chen et al. 2014), suggesting that the role of Msp1 in extracting mislocalized TA proteins is phylogenetically conserved. Upon extraction from the OMM, some of the TA proteins are rescued; they then travel to the ER, from where they continue to travel to their proper destination (Matsumoto et al. 2019). Others, when reaching the ER, are degraded by the ER-associated degradation (ERAD) machinery (Dederer et al. 2019, Matsumoto et al. 2019). Whether the TA proteins travel to the ER via a direct handover from Msp1 to the Get protein insertion machinery or via a different route is unknown. In summary, Msp1 surveys the OMM for mislocalized TA proteins and removes them from the membrane. This initiates either the redistribution of the TA proteins to their cognate destination organelles or degradation, thus achieving protein quality control to maintain cell health (**Figure 1**).

Msp1 was initially discovered nearly three decades ago in a screen for factors that sort proteins between the OMM and the IMM (hence the protein's name) (Nakai et al. 1993). However, efforts to validate the role Msp1 plays in this mechanism were not successful, suggesting that its overexpression in the screening regime may have artificially perturbed protein sorting. Only recently have the structure and function of Msp1 in protein quality control gradually come to light.

## 1.2. Msp1 as a Member of the AAA Family

Even though its function remained a mystery until recently, phylogenetic structural analyses (Frickey & Lupas 2004) indicated that Msp1 is a member of the large AAA protein family. There are about 20 different AAA proteins in yeast or human cells and about 60 in plant cells (Ogura & Wilkinson 2001). As their name suggests, they perform a wide variety of functions, such as degrading proteins (proteasome), disassembling SNARE complexes (NSF), and moving proteins across the membrane [cell division control protein 48 (Cdc48)]. Within this family, Msp1 clusters with katanin, spastin, Vps4, and fidgetin in one of six major subfamilies called the meiotic clade (MC). Recently, a number of high-resolution structures of AAA proteins (de la Peña et al. 2018, Dong et al. 2018, Gates et al. 2017, Han et al. 2017, Puchades et al. 2017, Twomey et al. 2019) have revealed a common mechanism of substrate unfolding: typically, the AAA protein forms a hexameric assembly and binds to its substrate in a central channel. The protein subunits undergo sequential ATP hydrolysis around the hexamer to generate mechanical forces that pull the substrate in two-amino acid steps through the channel, thereby unfolding it. Msp1 follows this general mechanism but in evolution has acquired special features that allow it to recognize and extract mislocalized membrane proteins.

Since the first reports on its activity in extracting mislocalized TA proteins, the field has made significant progress elucidating Msp1's mechanism of action. In Section 2 of this review, we summarize the current knowledge of the three major steps of the Msp1-mediated TA protein quality control pathway: (a) substrate recognition and recruitment, (b) substrate extraction, and (c) substrate degradation or resorting. We also discuss the structure and function of Msp1 within the context of the larger AAA protein family, and how its unique structural features are adapted to its function (Wang et al. 2020).



**Figure 1**

A model for Msp1-mediated protein quality control. This schematic figure shows two branches of the Msp1-mediated protein quality control pathways in distinct steps. (a) Msp1's mechanism of extracting mislocalized TA proteins. As denoted by gray arrows and step numbers, (1) Msp1 identifies and recruits the mislocalized TA proteins on the OMM, then (2) extracts them from the membrane. (3) The extracted TA proteins travel to the ER, possibly assisted by Get proteins or other chaperones. From there, (4) some of them (e.g., Gos1) continue to travel to their proper target organelle, while (5) others (e.g., Pex15 $\Delta$ 30) are shunted into a degradative pathway using the ERAD and proteasome machineries. (b) Msp1's mechanism of alleviating mitochondrial protein import stress. As denoted by the orange arrows and step numbers, (1) Msp1 is recruited to the TOM complex during stress conditions by the adaptor protein Cis1, where it extracts precursor proteins stuck in the TOM channel. (2) The extracted precursors are subsequently degraded by the proteasome. The route and mechanism by which the precursors reach the proteasome remain unknown (as indicated by the *double arrow*). Abbreviations: ATAD1, ATPase family AAA domain-containing 1; Cdc48, cell division control protein 48; Cis1, citrinin-sensitive knockout protein 1; ER, endoplasmic reticulum; ERAD, ER-associated degradation; Msp1, mitochondrial sorting of proteins 1; Npl4, nuclear protein localization protein 4; OMM, outer mitochondrial membrane; Pex15, peroxisomal membrane protein 15; TA, tail-anchored; TOM, translocase of the outer membrane; Ubc, ubiquitin-conjugating enzyme; Ufd1, ubiquitin fusion degradation protein 1.

### 1.3. Alleviating Mitochondrial Protein Import Stress

As discussed in the introduction to Section 1, the majority of mitochondrial proteins are encoded in the nucleus and imported to the mitochondria. Therefore, the normal function of the organelle relies on a functional protein import system. Under conditions in which the import machinery is

overwhelmed, several cellular pathways sense the stress and restore the organelle's protein import capacity. Among them is the mitochondrial compromised protein import response (mitoCPR) (Weidberg & Amon 2018). As a crucial part of mitoCPR, Msp1 is recruited to the TOM complex to clear precursor proteins that clog the translocase channel (Tom40), thus restoring its ability to import proteins (**Figure 1**). This discovery expanded the function of Msp1 from extracting mislocalized TA proteins to general quality control on the OMM. In Section 3, we review the current knowledge of Msp1's role in mitoCPR as well as its emerging role in general mitochondrial protein quality control.

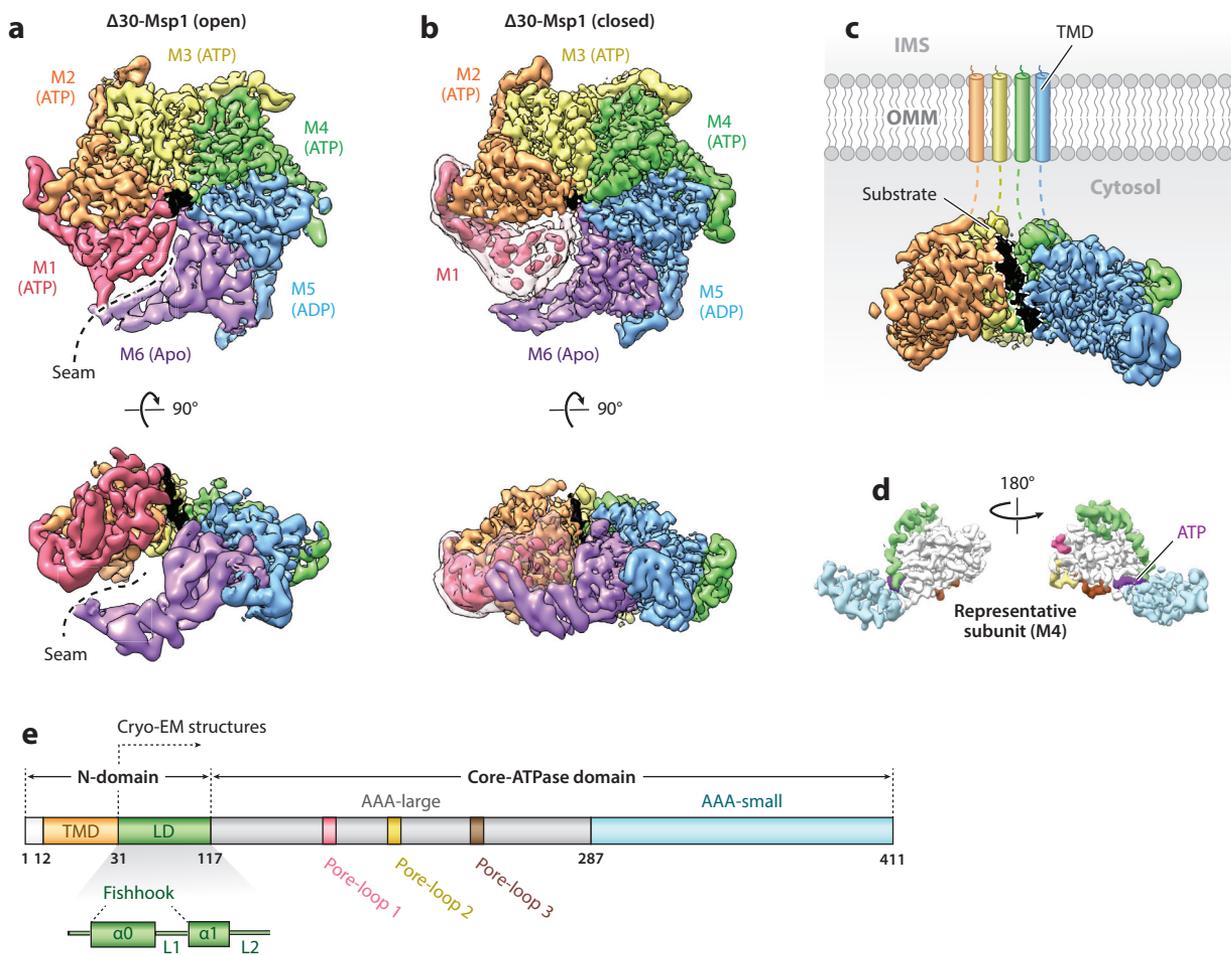
## 2. MECHANISM FOR EXTRACTING MISLOCALIZED TAIL-ANCHORED PROTEINS

As discussed in Section 1.1, Msp1/ATAD1 recognizes and extracts the mislocalized TA proteins for degradation or resorting. In this section, we synthesize the current knowledge about this process in three steps: (a) substrate recognition and recruitment, (b) substrate extraction, and (c) substrate degradation and resorting.

### 2.1. Substrate Recognition and Recruitment

The proper function of Msp1 depends on its ability to precisely distinguish endogenous mitochondrial TA proteins from mislocalized TA proteins, as extracting endogenous mitochondrial TA proteins (such as the small TOM complex proteins) could wreak havoc on the organelle. A major question remains in the field about how such discrimination is achieved. In addition, Msp1/ATAD1 localizes to both the peroxisomal membrane and the OMM (Chen et al. 2014, Okreglak & Walter 2014). Why does Msp1 only extract the Pex15 molecules that are mislocalized to the OMM and ignore those that normally exist on the peroxisome? What features of mitochondria-localized Pex15 mark it as foreign? In this section, we discuss the current models for Msp1 substrate recognition and the biological evidence for each of them. We approach this question from a structural point of view and discuss how the domain organization and structure of Msp1 shed light on its substrate-recognition mechanism.

**2.1.1. Current models for substrate recognition.** Several models have been proposed to explain how Msp1 selects its substrate. One model posits that a specific structural feature serves as a signal that renders a protein sensitive to Msp1 (which we termed the extracton signal), and that it is recognized by Msp1 directly in the substrate. Evidence for the extracton model comes from experiments using Pex15 $\Delta$ 30, a version of Pex15 in which 30 amino acids in its CTE have been removed, causing its mislocalization to mitochondria and degradation in an Msp1-dependent manner (Okreglak & Walter 2014). Based on using Pex15 $\Delta$ 30 as a model substrate, Msp1 was proposed to recognize a 12-amino acid hydrophobic sequence in the juxtamembrane region of the substrate (Li et al. 2019). A minimal construct containing only the hydrophobic sequence, the transmembrane helix, and the shortened CTE is degraded in an Msp1-dependent manner, whereas deletion of this patch from full-length Pex15 $\Delta$ 30 renders it insensitive to Msp1. The insertion of this segment into the juxtamembrane regions of the mitochondrial TA proteins (Fis1 or Gem1) rendered them sensitive to Msp1-dependent degradation, suggesting that the hydrophobic patch contains both essential and sufficient information for recognition by Msp1 (Li et al. 2019). On Msp1, *in vitro* cross-linking studies showed that hydrophobic amino acids contained in the N-terminal domain (N-domain) directly contact the substrate (Li et al. 2019). These amino acids are distributed throughout the N-domain: some are found in the intramembrane space (IMS), some in the transmembrane anchor, and others in the linker that follows (see **Figure 2e** for the



**Figure 2**

The structure of Msp1. Cryo-EM structures of (a) Msp1 (open conformation) and (b) Msp1 (closed conformation) complexes are shown in top and side views (PDB ID: 6PDW and 6PDY). Both structures lack the first 30 amino acids, which include the transmembrane domain of Msp1. Each subunit (from M1 to M6) is assigned a distinct color, and the substrate is shown in black. The nucleotide bound to each subunit is indicated in parentheses; Apo indicates that there is no bound nucleotide. The spiral seam of the open conformations is denoted with dashed lines. In panel b, the map for the mobile subunit M1 is depicted at two thresholds: solid pink for  $\sigma = 5.3$  (the same for the rest of the five subunits) and translucent pink for  $\sigma = 2.5$ , indicating that its weak density is due to the flexibility of this subunit and not to its complete dissociation from the complex. (c) Cutaway view of the open conformation revealing the substrate density (black; highlighted in white dashed lines) in the central pore. (d) A representative Msp1 subunit (M4) with domains and structural elements colored according to panel e. ATP is shown in purple. (e) A schematic illustration of the individual domains and structural elements of Msp1. The amino acid numbering is based on Msp1 from *Chaetomium thermophilum*. Abbreviations: cryo-EM, cryo-electron microscopy; IMS, intermembrane space; LD, linker domain; Msp1, mitochondrial sorting of proteins 1; N-domain, N-terminal domain; PDB, Protein Data Bank; TMD, transmembrane domain. Figure adapted from Wang et al. (2020).

domain organization). Mutations of these amino acids inactivate Msp1, causing a synthetic growth defect with  $\Delta get3$  (Li et al. 2019). Collectively, these results strongly indicate that the N-domain of Msp1 recognizes a special feature, an extracton signal, that is presented by a hydrophobic segment on the substrate. The extracton signal needs to be displayed in a particular geometry, because appending it to the N-terminus of Fis1 did not render Fis1 sensitive to Msp1 (Li et al. 2019).

In addition to the hydrophobic amino acids on Msp1, a single negatively charged amino acid (aspartate 12) residing in the IMS was found to interact with positively charged amino acids in the CTE of Pex15 $\Delta$ 30 (Li et al. 2019). This interaction adds to the strength of the substrate-Msp1 association yet does not provide the basis for discrimination between endogenous TA proteins on the OMM and mislocalized TA proteins, as many mitochondrial TA proteins have enriched positive charges in their CTEs.

The extracton model suggests that an interaction partner for Pex15 exists on the peroxisome that shields this Msp1-recognizable feature. Indeed, a separate study (Weir et al. 2017) showed that Pex3 interacts with Pex15 on the peroxisomal membrane and removing Pex3 results in the Msp1-dependent degradation of Pex15 on the peroxisome. Pex3 is a docking factor on the peroxisomal membrane important for peroxisomal protein import (Mast et al. 2020). The structure of Pex3 (Sato et al. 2010, Schmidt et al. 2010) shows that a cluster of hydrophobic amino acids is present in the membrane-proximal region that might shield the hydrophobic segment on Pex15. Thus, it is reasonable to infer from these two independent studies that Pex3 protects Pex15 from Msp1-mediated extraction through shielding its hydrophobic extracton signal.

A weakness of the extracton model is that it is currently based on studies of a single model substrate, Pex15 $\Delta$ 30, so its generality has not been established. Among the Msp1 TA protein substrates discovered so far [Pex15 (Chen et al. 2014, Okreglak & Walter 2014), Gos1 (Chen et al. 2014), Fmp32 (Dederer et al. 2019), Frt1 (Li et al. 2019), and Ysy6 (Li et al. 2019)], Pex15 is the only one with a recognizable hydrophobic segment at the juxtamembrane region. These TA protein substrates also do not possess recognizable common sequences or structural motifs. Therefore, other mechanisms for recognition are likely to exist.

The alternative but not mutually exclusive model hypothesizes that Msp1 selects its substrates based on their monomeric existence; i.e., Msp1 selects proteins that are not part of a larger protein complex. We termed this model the orphan model. By fusing oligomerization modules such as the FKBP or FRB domains to Pex15 $\Delta$ 30, researchers induced the dimerization of two Pex15 $\Delta$ 30 molecules and showed that, whereas the monomeric Pex15 $\Delta$ 30 molecules are efficiently degraded, artificially induced dimers are resistant to Msp1-mediated degradation (Dederer et al. 2019). In support of this model, the overexpression of Gem1 [a mitochondrial TA protein that is a component of the ER-mitochondria tethering complex ERMES (ER-mitochondria encounter structure) (Kornmann et al. 2009)] results in its Msp1-dependent degradation, as it outnumbers cognate complex partners (Dederer et al. 2019).

One explanation for these results is that the Msp1-recognizable feature in the substrate (such as the hydrophobic segment of Pex15 $\Delta$ 30) is occluded in a dimer or a higher-order protein complex. In this case, the extracton and orphan models converge on the same mechanistic explanation. An alternative explanation is that the extra transmembrane helices in a protein complex provide enough anchoring power to the membrane that it exceeds the pulling force of Msp1. In other words, Msp1 does not select its substrate based on a sequence motif or a structural feature but, rather, specificity is achieved based solely on the energy it takes to extract a protein from the lipid bilayer. Teasing out between the two models will require measuring Msp1's activity toward carefully designed substrates.

Based on the orphan model, all of the mitochondrial TA proteins would have to exist as part of bigger protein complexes (or oligomers) to evade extraction by Msp1. In yeast, there are six known mitochondrial TA proteins: Tom5, Tom6, Tom7, Tom22, Fis1, and Fmp32 (Burri & Lithgow 2004). Among those, the TOM proteins are firmly embedded in the TOM complex. Fis1 mediates mitochondrial fission and has known interaction partners, including Drp1 (Losón et al. 2013). Fmp32 is less well studied and is thought to be an assembly factor for cytochrome c oxidase (Paupe et al. 2015). This protein is rapidly degraded by Msp1 under physiological

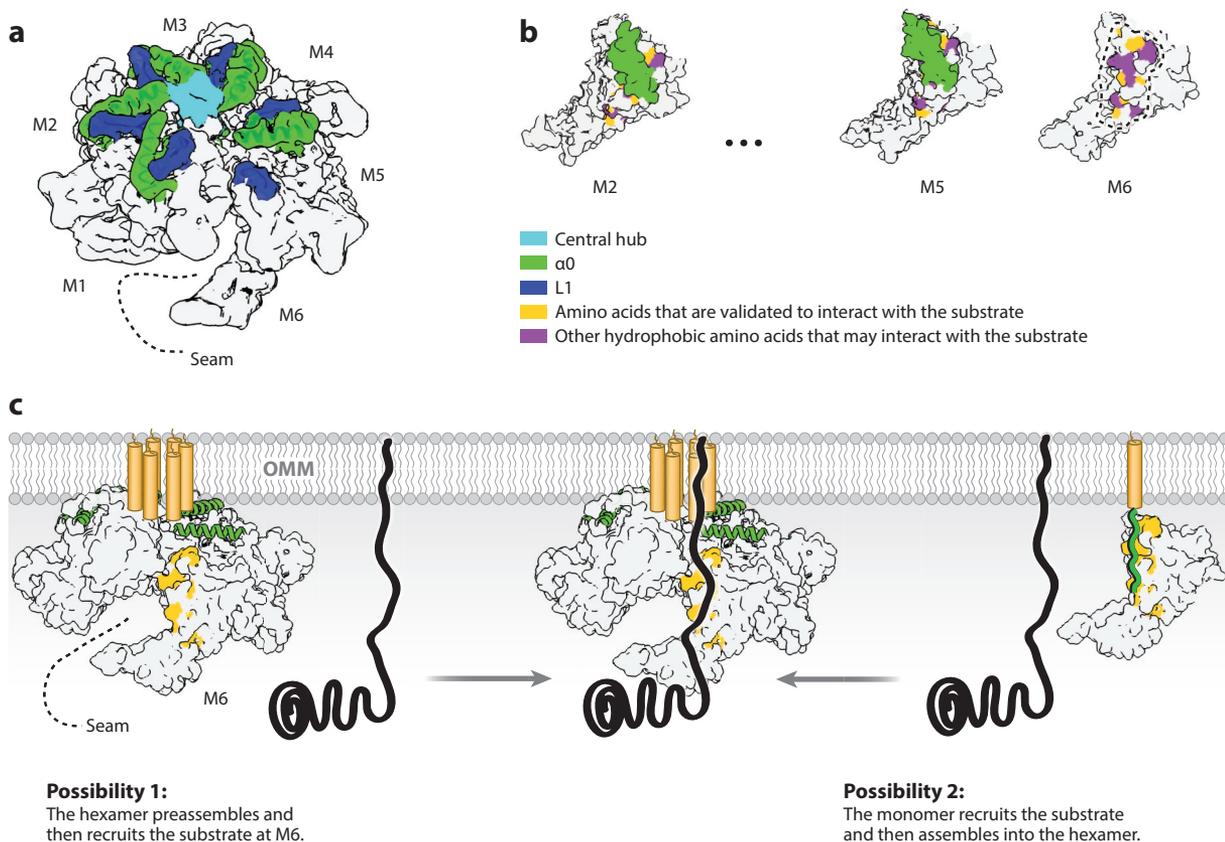
conditions, perhaps because it exists mainly in a monomeric state. If so, it would be interesting to ask whether inducing its dimerization would make it resistant to Msp1 extraction.

In addition to the functional evidence, the structure and domain organization of Msp1 provide further insight into its substrate recognition mechanism, which we review in detail next. In its role in relieving protein import stress, a completely different model for substrate recognition is proposed for Msp1: it is recruited by an adaptor protein to the TOM complex to extract the precursor proteins that clog the Tom40 channel. We discuss this process in Section 3.

**2.1.2. The structural basis for Msp1's substrate recognition.** A large number of mechanistic studies on AAA proteins (Augustin et al. 2009, Hanson & Whiteheart 2005) have provided us with a framework to understand the mechanism of Msp1. AAA proteins typically contain an N-domain that is responsible for substrate recruitment and selection followed by one or two classic AAA domains that form oligomers and harness the chemical energy of ATP hydrolysis to generate mechanical force. Unlike the typically highly conserved AAA domains, the N-domains show a large degree of variance among members of the AAA protein family, thus specializing each member for its particular function. In this section, we discuss the recently published cryo-electron microscopy (cryo-EM) structures of the Msp1-substrate complex with a focus on the N-domain, which sheds light on Msp1's substrate selection and recruitment mechanism. We also discuss the structure and domain organization of Msp1 in the context of the AAA protein family to delineate how its unique N-domain structure adapts the AAA domain engine to the special function of Msp1.

**2.1.2.1. The overall structure of the Msp1-substrate complex.** Recently, we solved a first set of structures of Msp1 in complex with a peptide substrate by cryo-EM (Wang et al. 2020). The structures contain the cytosolic domain of the *Chaetomium thermophilum* Msp1 lacking its N-terminal membrane anchor ( $\Delta 30$ -Msp1). Two different conformations were identified: an open conformation and a closed conformation (**Figure 2a,b**). In the open conformation,  $\Delta 30$ -Msp1 formed a homohexamer in which the six subunits (M1–M6) rotate and translate progressively to assemble into a right-handed open spiral with an open seam between the top (M1) and the bottom (M6) subunits, an assembly seen in many other AAA protein structures including katanin and spastin (**Figure 2a**). In the closed conformation, the Msp1 subunits assemble into the same spiral conformation, except for M1, which closes the seam of the spiral (**Figure 2b**). The cryo-EM density for M1 in the closed state is weak, indicating that the structure is an ensemble that represents a continuum of multiple states in the Msp1 reaction cycle. A 10-mer peptide was found in the central cavity formed by the hexamer that likely presents an averaged composite of many *Escherichia coli* peptides that copurified with Msp1 (**Figure 2c**). The well-resolved peptide backbone allowed us to closely examine the mechanism of substrate interaction in the central pore, which we discuss in detail in Section 2.2.

**2.1.2.2. The structure of Msp1's linker domain.** Each Msp1 subunit consists of an N-domain followed by a core-ATPase domain. In Msp1, the N-domain is composed of a transmembrane anchor (not included in our structures) followed by a linker domain (LD). The LD contains two helices ( $\alpha 0$  and  $\alpha 1$ ) and two loops (L1 and L2) (**Figure 2e**). Helix  $\alpha 0$  and loop L1 fold into a fishhook-shaped motif that is also observed in the structure of katanin (Zehr et al. 2017). In the open conformation, the  $\alpha 0$  helices in subunits M1–M5 are radially organized with their N-termini pointing toward the spiral's center, whereas the helix  $\alpha 0$  in M6 is disordered (**Figure 3a**). The N-terminal regions of the  $\alpha 0$  helices from M1 to M5 converge in a central hub, where they contact each other, with M1 being closest to and M5 farthest from the membrane. Helix  $\alpha 0_{M6}$  is unfolded, presumably because M6 occupies the lowest position in the spiral and therefore is positioned too



**Figure 3**

The structural basis for the extracton model of substrate recruitment. (a) A cryo-electron microscopy map of  $\Delta 30$ -Msp1 (open) showing the arrangement of the fishhook motifs in the spiral. Subunits M1–M5 show high density for the entire fishhook motif (helix  $\alpha 0$  and loop L1), whereas M6 shows density for L1 but not for  $\alpha 0$ . The structure shows the fishhook motifs of different subunits to be radially organized, with their N-termini pointing to the center of the spiral. (b) Surface representations of individual subunits highlighting amino acids in the linker domains (LDs) that are likely to engage with the hydrophobic substrate. These amino acids are buried by  $\alpha 0$  in M2–M5 but are exposed in M6 where  $\alpha 0$  is melted. (c) Two possible mechanistic explanations of the extracton model, shown in the outer mitochondrial membrane (OMM). (Left) In possibility 1, in the preassembled hexamer, the amino acids that interact with the substrate (identified in Li et al. 2019) by cross-linking or immunoprecipitation form a patch at the seam of the spiral, where they recruit the substrate using hydrophobic interactions. (Right) In possibility 2, monomeric Msp1 recruits the substrate and subsequently assembles into a hexamer. Figure adapted from Wang et al. (2020).

far away to participate in this stabilizing interaction at the central hub. The melting and refolding of  $\alpha 0$  allow the subunits to remain stably inserted in the membrane during Msp1's translocation cycle (a process discussed in detail in Section 2.2).

The conformational change of helix  $\alpha 0$  suggests a plausible mechanism for substrate recruitment by the extracton model, which posits that a series of hydrophobic amino acids in the N-domain of Msp1 directly engage the substrate Pex15 (Li et al. 2019). Mapping these amino acids to the structure shows that many of them lie in the LD. Intriguingly, they are only exposed at the surface in M6 [due to the melting of helix  $\alpha 0$  (Figure 3b)] but are mostly buried by the folded helices  $\alpha 0$  in M1–M5, suggesting that M6 is the only Msp1 subunit that exposes the possible substrate-binding site.

Binding could happen either before or after monomeric Msp1 assembles into a hexamer; that is, either the hexamer preassembles in regions where the local Msp1 concentration is high enough and then recruits the substrate at the binding site in M6 (**Figure 3c**), or, alternatively, Msp1 binds the substrate as a monomer. This latter notion is supported by the crystal structure of the monomeric *Saccharomyces cerevisiae* Msp1 (Wohlever et al. 2017), in which half of the LD is disordered, perhaps due to the lack of the stabilization interaction in the central hub present only in the oligomer. This suggests that helix  $\alpha 0$  may sample both the folded and the melted states in the monomeric form and thus could bind the substrate in its melted state and subsequently assemble into the M6 position of a hexamer (**Figure 3c**).

Interestingly, many of the hydrophobic amino acids in M6 line the open seam of the hexamer (**Figure 3c**). This arrangement could allow Msp1 to align the bound substrate with the seam, where it can be conveniently threaded into the central pore. Thus the observed conformational changes of helix  $\alpha 0$  provide an intuitive mechanism for substrate recruitment to Msp1's entry gate. Also, the membrane-proximal position of the LD of M6 provides an intuitive explanation for the geometric requirement that the extracton signal must be located next to the membrane.

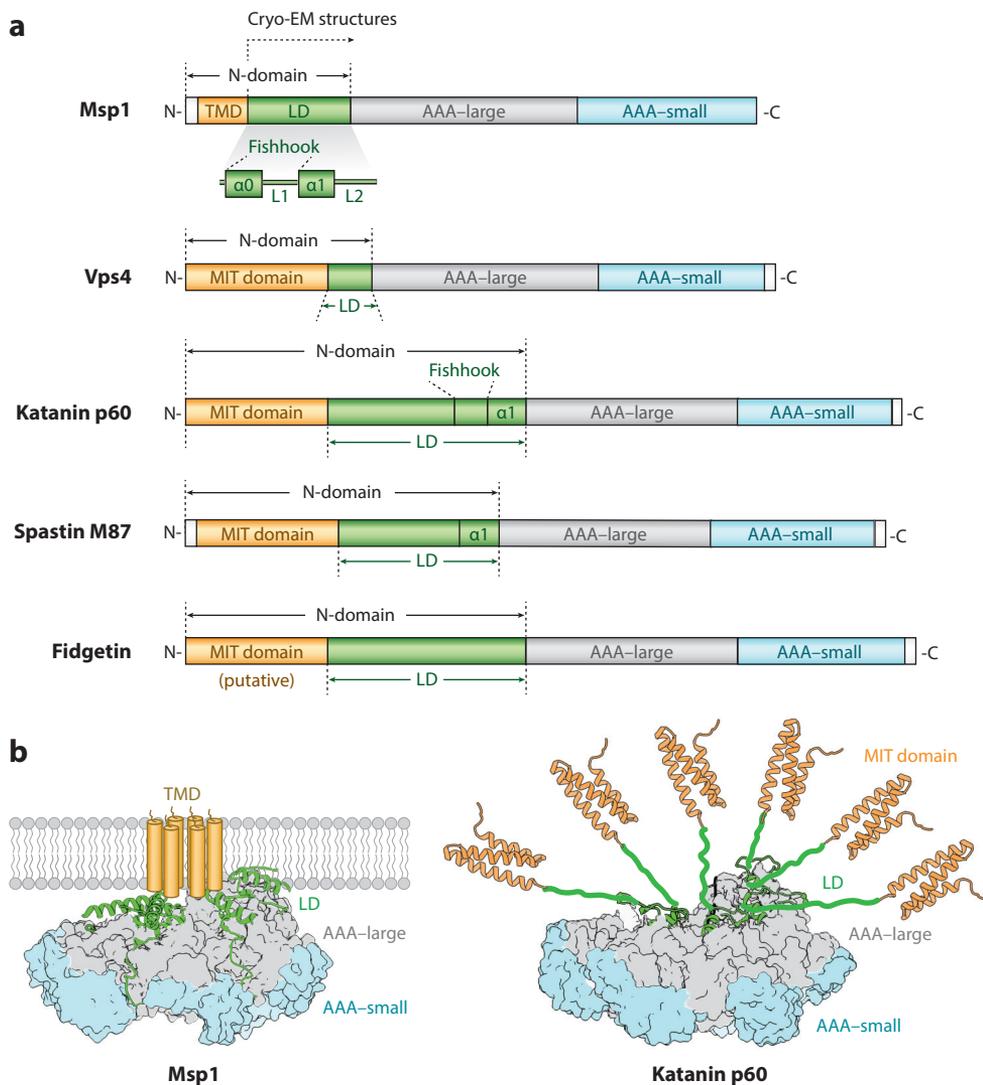
**2.1.2.3. A comparison of the N-domain of Msp1 to those of other AAA proteins.** According to the orphan model, Msp1 may lack intrinsic substrate specificity and could select its substrates purely on an energetic basis. A closer look at the N-domain of Msp1 provides support for this notion, as comparison of Msp1's N-domain to those of other AAA proteins has revealed some unique features. Within the AAA<sub>MC</sub> clade (**Figure 4a**), the N-domains of katanin, spastin, Vps4, and fidgetin all contain a microtubule-interacting and trafficking (MIT) domain that specifically interacts with their substrates [microtubules for katanin, spastin, and possibly fidgetin; ESCRT (endosomal sorting complexes required for transport) III peptides for Vps4]. Outside of the AAA<sub>MC</sub> clade, the N-domain of NSF mediates the interaction with its cofactor SNAP (soluble NSF attachment protein) proteins, which recruit substrate SNAREs; the N-domain of Cdc48 binds to cofactors ubiquitin fusion degradation protein 1 (Ufd1)/nuclear protein localization protein 4 (Npl4) that specifically interact with ubiquitin. By contrast, Msp1's N-domain lacks a module that targets it to a specific protein (**Figure 4a**).

Despite its simple makeup, the N-domain notably contains a transmembrane segment that limits Msp1's substrates to those embedded in or associated with the membrane to which Msp1 is attached. This is similar to a few other membrane-anchored AAA proteases such as FtsH and Yme1, both of which also appear to lack N-domains that would provide substrate specificity and which degrade a wide range of proteins present on the same membrane.

In summary, both the domain organization and the functional data suggest that the N-domain of Msp1 may not overly restrict its substrate recognition but rather may allow it to extract many different substrates localized to the OMM, some of which likely remain to be discovered. The main determinant of its substrate recognition may not be overly constrained by a sequence motif or structural feature but rather, as posited by the orphan model, may be primarily limited by the energy expense required to extract the protein from the membrane. Further insights will be aided by determining the biochemical and biophysical requirements for Msp1's substrate selection as well as by comprehensive proteomic analyses of the full spectrum of Msp1 substrates.

## 2.2. Substrate Extraction

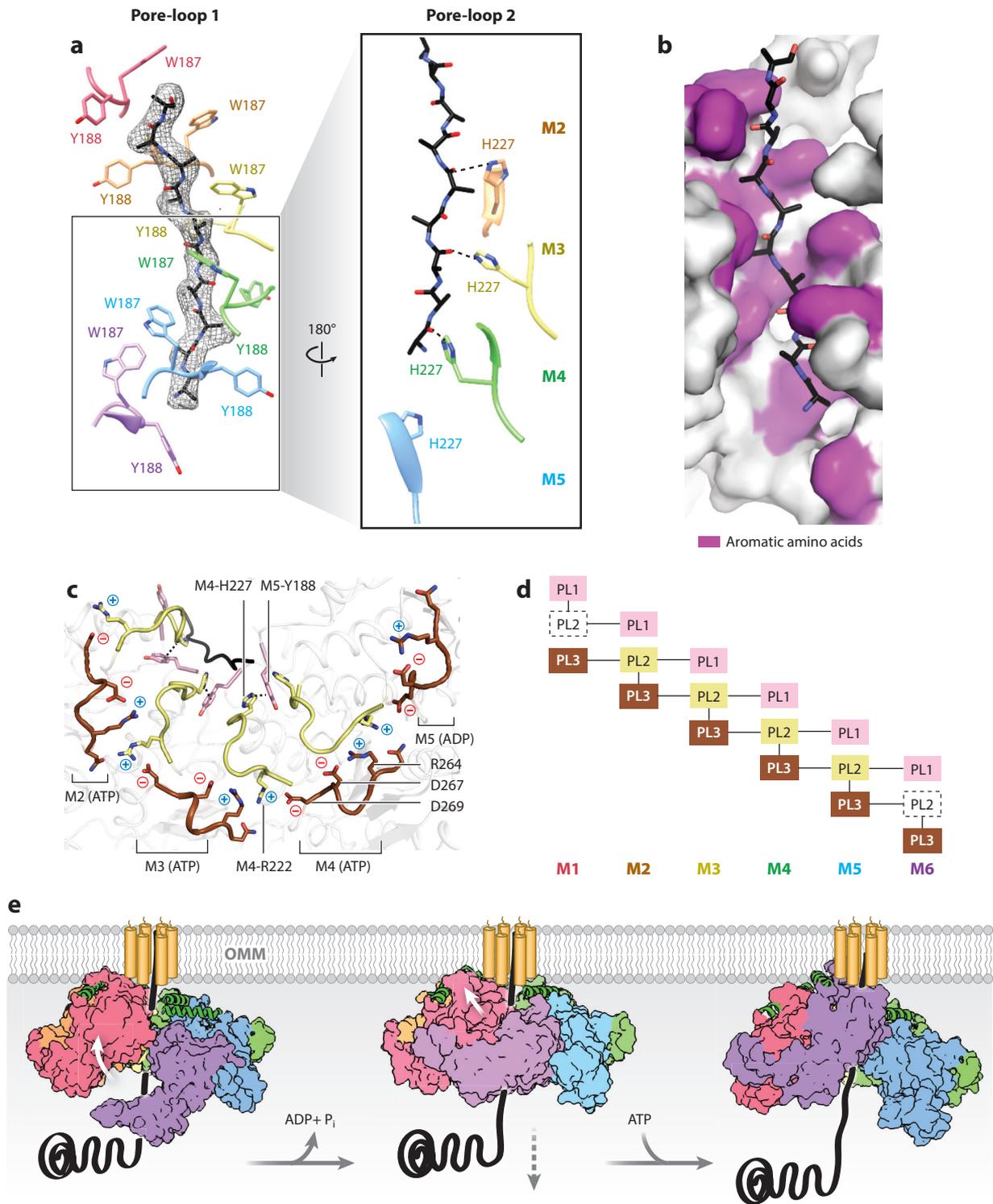
**2.2.1. The overall extraction mechanism.** Recent advances in single-particle cryo-EM have enabled the determination of many substrate-bound AAA protein structures at high resolution (Puchades et al. 2020). Many of these structures converge on a common hand-over-hand mechanism of protein unfolding: Upon recruitment, the substrate is threaded into the central



**Figure 4**

The domain organization of AAA<sub>MC</sub> proteins. (a) The domain organizations of the five major members of the AAA<sub>MC</sub> subfamily. The N-domains of Vps4, katanin, spastin, and fidgetin all contain an MIT domain, whereas in Msp1, the MIT domain is replaced by the TMD. (b) A surface representation showing the domain organization of Msp1 (PDB ID: 6PDW) and katanin p60 [PDB ID: 6UGD (core ATPase domain); PDB ID: 2RPA (MIT domain)]. Abbreviations: cryo-EM, cryo-electron microscopy; LD, linker domain; MC, meiotic clade; MIT, microtubule interacting and trafficking; Msp1, mitochondrial sorting of proteins 1; N-domain, N-terminal domain; PDB, Protein Data Bank; TMD, transmembrane domain.

pore, a narrow channel lined by the AAA protein hexamer. Each subunit extends one or two pore-loops (pore-loops 1 and 2) toward the center of the pore to grip the substrate (**Figure 5a**). By contrast, residues contributed by pore-loops 3 also line the pore but do not interact directly with the substrate. The subunit at the terminal position (M6) of the spiral, that is, the one at the seam, hydrolyzes ATP, loosens its grip on the substrate and leaves its firm connection to the spiral. Upon loading of a new ATP molecule, M6 reattaches to the spiral at the top position (now becoming



(Caption appears on following page)

**Figure 5** (Figure appears on preceding page)

Substrate extraction mechanism. (a) Pore-loops 1 (PL1s) form a spiral staircase around the substrate (with peptide density shown as a *black mesh*). PL2s form a second staircase below PL1s. H227s form hydrogen bonds with the peptide backbone carbonyls (*dashed lines*). (b) A surface representation of the central pore showing that the peptide (in *stick representation*) is surrounded by interdigitating aromatic amino acids (*purple*) in the central pore. (c) An illustration of the PLs showing that PL2s interact with PL1s through  $\pi$ - $\pi$  stacking and with PL3s via electrostatic and polar interactions. The  $\pi$ - $\pi$  stacking interaction is shown with dashed lines, and the positive charges are shown in blue while negative charges are in red. PL1s are shown in pink, PL2s in light yellow, and PL3s in brown. (d) A schematic diagram showing the interactions (depicted by *lines*) between PLs. The disordered PL2s in M1 and M6 are shown in dashed boxes. (e) A model for substrate extraction showing the upward translocation of the bottom subunit (M6) and the downward translocation of the substrate through the central pore. A tail-anchored (TA) protein substrate is shown in black. The Msp1 (mitochondrial sorting of proteins 1) subunits are shown in surface representation and are colored as in **Figure 2**. The folded  $\alpha 0$  helix is shown in cartoon representation (*green*) and the positions of the N-terminal transmembrane regions are schematically indicated. Figure adapted from Wang et al. (2020).

M1), where its pore-loops reengage the substrate (**Figure 5e**). Each upward translocation event like this allows the protein to move along the peptide substrate in two amino acid steps, and, in Msp1's case, to extract the substrate stepwise out of the membrane. During the movement of the terminal subunit, pore-loops from the other subunits remain engaged with the substrate, preventing it from backsliding. The pore-loops hold the substrate in an almost linearly extended conformation imposed by the shape of the central pore. In this way, Msp1 (and AAA proteins in general) effectively unfolds its substrate as it traverses the pore.

**2.2.2. The structural features that adapt Msp1 to its function.** Msp1 follows the conserved general mechanism of protein unfolding established for AAA proteins, yet several structural features specialize it for its unique role of extracting transmembrane proteins from the lipid bilayer. First, the  $KX_1X_2G$  motif in pore-loop 1 is highly conserved across the entire AAA protein family, with  $X_1$  being an aromatic amino acid and  $X_2$  usually a nonaromatic hydrophobic amino acid. The aromatic amino acid intercalates between the substrate's side chains and form a staircase around the substrate. By contrast, in Msp1/ATAD1, both  $X_1$  and  $X_2$  are aromatic amino acids (Msp1: W187 and Y188), and both are in direct contact with the substrate's side chains (**Figure 5a**). Second, pore-loop 2 is highly variable across the AAA protein family and is usually disordered or retracted from the substrate. By contrast, the pore-loops 2 of Msp1 are in close contact with the substrate, forming a second staircase around the substrate just below pore-loops 1. In addition, a conserved histidine (Msp1: H227) forms a hydrogen bond with the backbone carbonyl of the substrate (**Figure 5a**). In total, not just one but three aromatic amino acids from each subunit tightly engage the substrate (**Figure 5b**), making the central pore of Msp1 more hydrophobic and sterically constrained than those in other AAA proteins.

A recent study (Rodriguez-Aliaga et al. 2016) on the ClpXP motor showed that the bulkiness of the pore-loops positively correlates with their grip on the substrate but inversely correlates to the substrate pulling velocity. As Msp1 needs to extract the hydrophobic membrane anchors from the lipid bilayer, it would seem beneficial for it to exert a stronger grip on its substrate using bulky pore-loops to prevent backsliding. Similar to Msp1, other AAA proteins that extract membrane proteins, such as Cdc48 (Cooney et al. 2019, Twomey et al. 2019), Yme1 (Puchades et al. 2017), and AFG3L2 (Puchades et al. 2019), all have second aromatic amino acids in addition to the conserved ones in pore-loops 1 that directly interact with the substrate, suggesting that distantly related AAA proteins may have converged on similar solutions to increase their grip on membrane protein substrates.

In addition to providing extra grip, the pore-loop 2 of Msp1 is also uniquely positioned to sense the position of the subunit in the spiral. It is involved in an interconnected network of electrostatic interactions involving pore-loops 1, 2, and 3 (**Figure 5c,d**). Its stabilization requires interactions

from subunits on both sides. As a result, pore-loops 2 in the central four subunits (M2–M5) are ordered, and those in subunits capping the spiral (M1 and M6) are disordered due to the lack of an interaction partner on opposite sides of the seam (**Figure 5d**). This allows M6 to sense its bottom position in the spiral and initiate its dissociation from the substrate. The importance of the amino acids in all three pore-loops has been validated experimentally (Wang et al. 2020).

In addition to the sophisticated network of pore-loop–pore-loop interactions, Msp1 uses a unique mechanism for communication between subunits. A few AAA proteins (such as Yme1 and those found in the cap structure of the proteasome) use an intersubunit-signaling (ISS) motif that contains a signature DGF tripeptide to sense the nucleotide state and transmit this information to the pore-loops. In Msp1, as in other AAA<sub>MC</sub> proteins, the crucial phenylalanine in the ISS motif is replaced by an aliphatic amino acid, which is predicted to hamper transmission of the information on nucleotide state by this mechanism. Instead, Msp1 possesses a short loop insertion C-terminal to the traditionally defined ISS motif [the nucleotide communication loop (NCL)] that senses the nucleotide hydrolysis and communicates it to the adjacent subunit. Together with the melting of pore-loop 2 when it reaches the M6 position, the NCL prompts the terminal subunit for its upward translocation. Although this loop seems to be unique to the *C. thermophilum* Msp1 and is not conserved in the Msp1 family, it is also found in similar proteins such as katanin. Whether it also plays a role there in the transmission of nucleotide states remains to be determined.

In summary, while following the general hand-over-hand mechanism, Msp1 has acquired a few structural modifications to the conserved AAA fold. These modifications are uniquely suited to its role as a membrane protein extractase.

Despite the rich mechanistic insights provided by these structures, several questions remain. First, a recent study (Han et al. 2019) showed that the central pore of Vps4 is able to engage a circular peptide, an indication that it could unfold internal sequences. Can Msp1 also hold two polypeptide chains in its pore at once and hence unfold internal sequences that are part of a folded structure? The structure of Msp1 shows that the bulky pore-loops encase the substrate tightly and leave almost no space to fit another peptide. If Msp1's central pore can be shown to similarly accommodate an extra peptide chain, what conformational changes are required for this to happen? Second, given that the NCL is not an evolutionarily conserved feature across the Msp1/ATAD1 subfamily, what mechanism is employed by the Msp1 orthologs to communicate the nucleotide state between subunits? Lastly, does Msp1's transmembrane helix play a role in substrate extraction beyond aligning the extractase on the surface of the membrane?

### 2.3. Substrate Degradation or Resorting

While a significant amount of work has expanded our understanding of how Msp1 selects and extracts its substrates, the fate of the extracted TA proteins has remained a mystery until very recently. Previous studies (Chen et al. 2014, Okreglak & Walter 2014) showed that mislocalized TA proteins are proteolytically degraded upon Msp1 extraction. Given that Msp1 itself does not have a protease function, the degradation must require the channeling of the extracted protein to the degradative machineries. A recent study (Matsumoto et al. 2019) showed that the degradation of the Msp1 substrates is proteasome mediated. To identify factors involved in this process, the authors conducted a limited screen of the ubiquitin–proteasome-related genes that revealed that the ER-resident E3 ubiquitin ligase Doa10, its associated E2 ubiquitin-conjugating enzymes Ubc6 and Ubc7, and their tethering factor Cue1 are all required for the degradation of Pex15Δ30. In addition, Cdc48 and its cofactors Ufd1, Npl4, and the tethering factor Ubx2 are also required for its degradation. Interestingly, these proteins constitute the cytosolic branch of the ERAD pathway (ERAD-C), which degrades the ER-membrane proteins with misfolded cytosolic domains. The

surprising finding that the ERAD machinery is required for the degradation of Msp1 substrates indicates that Msp1 drives the substrates from the OMM to the ER membrane (**Figure 1**). In line with this notion, the authors (Matsumoto et al. 2019) showed that the deletion of Msp1 from cells lacking a functional ERAD-C machinery ( $\Delta ubc7$ ) leads to the accumulation of Pex15 $\Delta$ 30 on the OMM, whereas the overexpression of Msp1 in these cells leads to its enrichment on the ER. In contrast to Pex15 $\Delta$ 30, another Msp1 substrate, mitochondrially mislocalized Gos1, escapes degradation by ERAD and ends up on the Golgi apparatus, its cognate organelle of residence. These observations are explained by the notion that Pex15 $\Delta$ 30 is an artificial protein that is seen by the ERAD system as foreign, whereas Gos1 is an endogenous protein whose targeting to the ER is on pathway during its normal biogenesis, therefore allowing it to escape the degradation. An alternative, albeit less interesting, explanation is that during extraction, Msp1 unfolds part of its substrates, and the observed differences are due to different refolding efficiencies of different substrates: Pex15 $\Delta$ 30 may remain partially unfolded by the time it reaches the ER, which causes the molecule to be marked by ERAD-C as a defective protein substrate ready to be degraded, whereas Gos1 may refold by the time it reaches the ER (perhaps due to its strong helical propensity) and thus escape degradation.

The involvement of ERAD-C was independently shown by a separate study (Dederer et al. 2019) in which researchers used the fluorescent timer strategy to identify genes whose absence leads to a longer half-life of the substrate. From a genome-wide screen and follow-up experiments, the authors also determined that the Doa10 complex is responsible for degrading Pex15 $\Delta$ 30. In addition, the researchers showed that a small fraction of TA protein substrates are degraded by the vacuole.

These two independent studies unambiguously showed that the ERAD-C pathway plays an important role in the degradation of Msp1 substrates. The requirement for the collaboration of two independent quality control systems on two separate membranes to degrade the mislocalized TA proteins remains surprising. The possibility to correct mistakes by sending mislocalized proteins such as Gos1 back to the ER may offer an explanation. How proteins are shuttled from Msp1 to the ER still remains a mystery, as proteins containing transmembrane sequences are unlikely to diffuse through the cytoplasmic space unchaperoned. Msp1 extraction may therefore be obligatorily coupled to a transport system, perhaps hitchhiking on the Get machinery, to reach the ER. Alternatively, Msp1 may release its substrates at ER-mitochondria contact sites to allow direct delivery. Nevertheless, ERAD-C and the proteasome complete the Msp1-assisted protein quality control pathway.

### **3. THE ROLE OF Msp1 IN ALLEVIATING MITOCHONDRIAL PROTEIN IMPORT STRESS**

All but a few mitochondrial proteins are encoded in the nuclear genome and thus need to be imported across the mitochondrial membranes. Together the multicomponent translocase of the outer membrane (TOM) and that of the inner membrane (TIM) constitute one of the main mitochondrial protein import machineries. Typically, a protein is targeted to the TOM complex by a short peptide at the N-terminus [the mitochondrial targeting sequence (MTS)] (Neupert & Herrmann 2007). Powered by the electrochemical potential across the IMM and ATP hydrolysis, the protein traverses the TOM and then the TIM channels and finally emerges in the matrix space. In the cases in which the protein possesses an internal hydrophobic sequence, it stalls the transfer in the TIM23 channel, from which it is laterally released into the IMM. Maintaining unimpeded flow in the import channels is essential, as the clogging of these channels not only leads to a failure in obtaining the necessary proteins for the organelle's function but also causes the accumulation of

unimported precursor proteins in the cytosol. Such accumulation sequesters essential chaperones and leads to a failure of protein homeostasis. Despite the severe outcome, until recently little was known about how the organelle protects itself in the face of such import stress.

The mitoCPR protects the mitochondria when the import machinery is overwhelmed. The overexpression of proteins containing both the N-terminal MTS and an internal hydrophobic sequence (a bipartite signal) leads to the TIM23 channel becoming clogged due to the slow lateral release of the hydrophobic sequence into the IMM. This defect consequently causes a clog in the TOM channel, preventing more proteins from being imported and processed. In response to this import stress, cells activate the mitoCPR, which is mediated by the transcription factor Pdr3.

As a crucial part of the mitoCPR, Msp1 is recruited to the TOM complex by Cis1. *CIS1* is one of the most strongly induced genes in response to *PDR3* activation. It accumulates at the surface of the mitochondria under import stress. Upon recruitment, Msp1 extracts the precursor proteins from the import channel, and they are degraded in a proteasome-dependent manner (**Figure 1**). Consequently, Cis1 and Msp1 together restore the ability of the mitochondria to import proteins during the period of import stress. Mechanistically, it is still a mystery how Msp1 gains access to the precursor proteins stuck in the Tom40 channel. Whether Cis1 or other factors are required to help shuttle these precursors to Msp1 remains to be understood.

The function of Msp1 in mediating protein import is apparently independent from its function in extracting mislocalized TA proteins. Overexpressing Cis1 in the  $\Delta get3$  background causes a growth phenotype resembling the  $\Delta get3/\Delta msp1$  double knockout, indicating that Msp1 is redistributed to the TOM complex by binding to Cis1, which precludes it from extracting TA proteins.

Msp1's function in assisting with mitochondrial protein import stress is supported by several lines of evidence. First, *MSP1* has displayed a strong genetic interaction with several genes encoding subunits of the TOM complex under respiratory conditions: *TOM5*, *TOM20*, and *TOM40* (Basch et al. 2020). Both Tom5 and Tom20 are known to bind incoming precursor proteins (Bausewein et al. 2017, Söllner et al. 1989), and their absence leads to the accumulation of improperly channeled precursor proteins at the main import channel (Tom40). Second, in vitro import assays show that Msp1 is required for the removal of mitochondria-targeted DHFR, a model substrate whose core can be stabilized by methotrexate and then is particularly hard to unfold, thus clogging the import channel (Basch et al. 2020). Finally, the overexpression of Msp1 leads to reduced levels of several IMS proteins whose import is less efficient, suggesting Msp1 may actively remove proteins that do not easily pass the channel (Basch et al. 2020). In summary, by extracting proteins that are stuck in the import channel, Msp1 clears the path for more precursor proteins and alleviates the mitochondrial import stress.

Upon extraction by Msp1, the precursor proteins are degraded in a proteasome-dependent manner (Basch et al. 2020); however, the route by which the substrate proteins reach the proteasome remains ill defined. Immunoprecipitation followed by mass spectrometry (IP-MS) showed that the proteasomal subunit Rpn10 interacts with Msp1 under nonstress conditions (Basch et al. 2020), suggesting that extracted precursor proteins are directly handed over to the proteasome. This suggests that precursor protein ubiquitylation is coordinated with Msp1 extraction at the OMM, which is different from that of TA proteins that become ubiquitylated at the ER membrane after Msp1 extraction (Dederer et al. 2019). However, a similar IP-MS experiment described previously (Chen et al. 2014) failed to uncover any proteasomal subunits, perhaps due to different Msp1 expression and enrichment methods. A direct proteasomal association thus requires further validation, and the ubiquitylation machinery required for the degradation remains to be defined.

Finally, how cells sense the protein import stress and relay that information from the mitochondria to the nucleus remains a mystery. Recently, the activation of Pdr3 was shown to be the last step of a reaction cascade: it begins with the activation of Hsf1 (Boos et al. 2019), the key transcription

factor driving the heat shock response. Hsf1 is normally repressed by binding to molecular chaperones and is activated when the chaperones are sequestered by unimported precursor proteins. Upon activation, Hsf1 induces the expression of a proteasomal subunit Rpn4, a transcription factor that in turn induces the expression of Pdr3. Thus, mitoCPR is part of a multilayered cellular response to mitochondrial protein import stress. Whether Pdr3 can be activated by a mechanism independent of Rpn4 or Hsf1, that is, whether it can sense the import stress directly, and what role Msp1 plays in this process remain open questions.

Likewise, whether Msp1/ATAD1 is involved in protein import quality control in higher eukaryotes remains an open question. Although Cis1 does not have an apparent ortholog in mammalian cells, a different adaptor protein could recruit Msp1 to the TOM complex. This is supported by the fact that Cis1 deletion only has a mild effect on mitoCPR in yeast (Weidberg & Amon 2018). Alternatively, Msp1 could have an intrinsic affinity to the TOM complex and the adaptor(s) may enhance binding during import stress. Interestingly, a loss-of-function mutation in the *Caenorhabditis elegans* Msp1 (Mspn-1) causes the upregulation of the ATFS-1 transcription factor (Basch et al. 2020), which is a hallmark of the mitochondrial unfolded protein response (UPR<sup>mt</sup>).

Whereas Msp1's essential role in facilitating mitochondrial import under stress is well established, its role under nonstress conditions is less well defined. A recent study (Mårtensson et al. 2019) described a protein quality control pathway termed mitoTAD (mitochondria translocation-associated degradation). Under normal conditions, the soluble AAA protein Cdc48 is recruited to the OMM by its adaptor Ubx2 to remove proteins that stall the TOM complex and extract them for degradation. The study showed that the parallel deletion of *UBX2* and *MSP1* causes the synthetic growth defect in yeast, suggesting that Msp1 has a role in a pathway parallel to mitoTAD. Although under the described experimental conditions Msp1 is not pulled down with Tom40, its involvement in protein import control has not been ruled out.

Finally, a separate study (Chen et al. 2014) identified a number of ribosomal subunits that are enriched in pull-downs of Msp1<sup>E193Q</sup> in wild-type cells but not in  $\Delta get3$  cells, suggesting that Msp1 might associate with ribosomes at the mitochondrial surface under normal conditions, potentially engaged in some form of cotranslational protein quality control, and is repurposed to extract TA proteins when their mislocalization is induced. A comprehensive identification of the full set of Msp1 substrates and interacting partners under nonstress and stress conditions promises to shed further light on the functional scope of Msp1 activity.

#### 4. ATAD1 AND THE REGULATION OF SYNAPTIC ACTIVITIES

Msp1 is highly conserved across eukaryotic species. The yeast Msp1 shares a high degree of similarity with its mammalian homolog ATAD1 (also named Thorase after Thor, the hammer-wielding Norse god of thunder, in reference to its proposed function of being a molecular hammer that breaks down receptor protein complexes), with the key amino acids in the pore-loops and the nucleotide-binding pocket strictly conserved (Wang et al. 2020). Functionally, in addition to extracting mislocalized TA proteins from the OMM, recent studies (Dai et al. 2010; Zhang et al. 2011a,b) have established ATAD1 as a neuroprotective gene, although compared to its role in mitochondrial protein quality control, the mechanistic details of its neuroprotective activities are less well understood.

ATAD1 emerged as a hit from a two-step functional screen for genes whose expression were (a) induced in neurons during sublethal oxygen-glucose deprivation (OGD) conditions and (b) cytoprotective when cells were subjected to a subsequent, more severe oxidative stress (Dai et al. 2010). Multiple lines of evidence confirmed ATAD1's neuroprotective function: in neurons,

the knockdown of *ATAD1* resulted in a decreased survival rate during OGD, whereas its overexpression increased the survival rate during OGD or excitotoxic exposure to the neurotransmitter N-methyl-D-aspartate (NMDA) (Dai et al. 2010). In animals, mice overexpressing *ATAD1* had fewer neurological deficits after a stroke than their wild-type counterparts, whereas *ATAD1*<sup>-/-</sup> mice showed enhanced neurological deficits and eventually died from seizure-like syndromes. In humans, exome sequencing of patients with familial neurological disorders such as schizophrenia and encephalopathy revealed a strong correlation between *ATAD1* mutations and these diseases (Piard et al. 2018, Umanah et al. 2017).

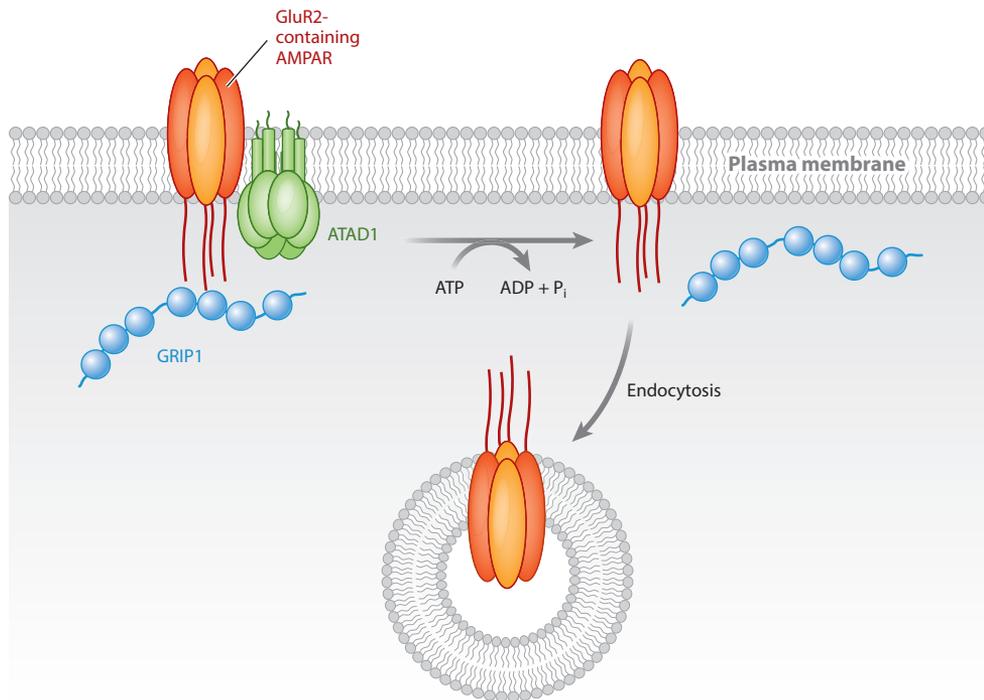
The prevailing theory to explain *ATAD1*'s neuroprotective function is that it works through regulating receptor trafficking at the synapse, an activity that is seemingly independent of its function in mitochondrial protein quality control (Zhang et al. 2011b). In particular, *ATAD1* is thought to regulate the trafficking of the AMPA receptors (AMPA), the most ubiquitously present neurotransmitter (glutamate)-gated ion channel in the nervous system. The number of AMPARs displayed at the cell surface determines, in part, the strength of the synapse and is dynamically regulated by the receptors' insertion into and removal from the postsynaptic membrane. The dysregulation of these processes underlies many neurological disorders, including schizophrenia (Rubio et al. 2012) and epilepsy (Rogawski 2013). Multiple lines of evidence suggest that *ATAD1* frees the AMPAR from its interaction partner GRIP1 (glutamate receptor-interacting protein 1), a scaffolding protein that stabilizes AMPAR at the postsynaptic membrane, thus licensing the endocytic removal of the AMPARs (**Figure 6**) (Zhang et al. 2011b). These lines of evidence are as follows:

1. Immunoprecipitation experiments in both HEK293 cells and mouse brain lysates showed that GluR2, one of the four subunits that constitute the tetrameric AMPAR, forms a complex with *ATAD1* and GRIP1;
2. In vitro reconstitution assays with purified components showed that *ATAD1* could disassemble the GluR2-GRIP1 complex in an ATP-dependent manner; and
3. In neurons, the overexpression of *ATAD1* (but not *ATAD1* bearing mutations that inactivated its ATPase activity) caused a decrease in the surface AMPAR level, whereas its deletion led to an increase in surface-displayed AMPARs.

*ATAD1* not only regulates the steady state level of surface AMPARs under normal conditions but also is required for the active endocytosis of these receptors in response to a stimulus. When treated with bicuculline, a molecule that inhibits the GABA<sub>A</sub> receptor (an inhibitory neurotransmitter receptor), wild-type neurons internalized a significant portion of AMPARs (excitatory receptors) from the cell surface. This process, which is called synaptic downscaling, allows neurons to adjust the levels of neuroreceptors to retarget their baseline activity properties in the face of a continued perturbation. In *ATAD1*<sup>-/-</sup> neurons, however, bicuculline treatment did not affect the surface AMPAR level, indicating that *ATAD1* is required for synaptic downscaling (Zhang et al. 2011b).

In addition to the biochemical and cell biological evidence mentioned earlier in this section, the function of *ATAD1* in regulating AMPAR trafficking is also supported by pharmacological evidence. The treatment of *ATAD1*<sup>-/-</sup> mice as well as patients with *ATAD1* mutations with an AMPAR antagonist was found to mitigate neurological defects (Ahrens-Nicklas et al. 2017).

The proposed mechanism of action for *ATAD1* to unravel the binding interaction between GluR2 and GRIP1 closely resembles that of the AAA protein NSF, which has been extensively characterized for its role in disassembling SNARE complexes to free up SNARE proteins for reuse. Similar to the function of *ATAD1*, NSF disrupts the interaction between GluR2 and its interaction partner PICK1 in an ATP-dependent manner. However, unlike GRIP1, PICK1 facilitates the



**Figure 6**

A model for the regulation of AMPAR trafficking by ATAD1. This model shows a possible mechanism for the activity of ATAD1 (ATPase family AAA domain-containing 1) in regulating AMPAR endocytosis. ATAD1 binds to the C-terminal tail of the GluR2-containing AMPAR and disassembles it from GRIP1 (glutamate receptor-interacting protein 1) in an ATP-dependent manner. This reaction frees up the GluR2-containing AMPARs and allows them to be removed from the postsynaptic membrane through endocytosis. ATAD1 is shown here with its transmembrane regions inserted into the plasma membrane, which is a conjecture that has not been experimentally validated.

endocytic removal of GluR2. Therefore, the activity of NSF helps to stabilize the AMPARs at the postsynaptic membrane (Hanley et al. 2002).

The comparison to NSF reveals a major mechanistic paradox for the mechanism of ATAD1. While NSF is a soluble protein that can approach AMPARs from the cytosol, how ATAD1, a known organelle membrane protein, could perform this task is unclear. Analyses in several cell lines confirm that human ATAD1 localizes to both the OMM and the peroxisomal membrane, as does its yeast ortholog Msp1 (Chen et al. 2014). One can envision several possibilities for how ATAD1 might interact with AMPAR on the plasma membrane. Perhaps a fraction of ATAD1 in neurons localizes to the postsynaptic plasma membrane, allowing it to approach its substrates in a mechanism similar to that described in the TA protein extraction: the cytosolic region of GluR2 binds ATAD1 and is threaded into the central pore, where it is linearized and stripped off from its interaction partner GRIP1. Alternatively, there may be a cytosolic pool of ATAD1 in neurons from which the enzyme could approach its substrate, analogous to the way in which NSF interacts with the AMPAR. In line with this notion, one study (Chen et al. 2014) showed that when overexpressed in HeLa cells, ATAD1 is partially cytosolic, perhaps mimicking the high

ATAD1 expression level in the brain. We note that ATAD1's N-terminal transmembrane domain is only mildly hydrophobic, adding plausibility to this scenario as it may weaken ATAD1's membrane association and allow it to exist in equilibrium between soluble and membrane-anchored states.

In addition to its proposed role of directly catalyzing the dissociation of GluR2 from GRIP1, ATAD1's neuroprotective activity may result from its role in mitochondrial quality control. The deletion of *ATAD1* in mice causes mitochondrial fragmentation and mitochondrial protein loss in the brain (Chen et al. 2014), suggesting that it is critical to maintaining mitochondrial function. As mitochondrial dysfunction can cause neurological diseases such as Parkinson's disease (Sekine & Youle 2018), the major neurological defects observed in animals and patients with defective ATAD1 may be attributed, at least partially, to ATAD1-related mitochondrial damage.

## 5. CONCLUDING REMARKS

Cells spend a significant amount of energy to maintain protein homeostasis. Over the past few years, a combination of genetic, cell biological, biochemical, and structural studies have established Msp1 as a key regulator of mitochondrial protein homeostasis. Since the initial discovery of its function (Chen et al. 2014, Okreglak & Walter 2014), the field has made considerable advances in understanding the mechanism of Msp1-mediated membrane protein extraction. The first advance involved identifying how Msp1 distinguishes the substrate from the nonsubstrate, that is to say, how Msp1 acts precisely on the mislocalized proteins and ignores ones that are correctly targeted to the OMM (Dederer et al. 2019, Fresenius & Wohlever 2019, Li et al. 2019, Weir et al. 2017). The second advance was examining how Msp1 overcomes the energetic barrier of extracting hydrophobic sequences from the greasy lipid bilayer (Wang et al. 2020, Wohlever et al. 2017). The third advance was tracking the fate of the extracted TA proteins and identifying the other players involved in this pathway (Dederer et al. 2019, Matsumoto et al. 2019). Going beyond TA protein extraction, the discovery of the mitoCPR pathway in yeast expanded the function of Msp1 to include the regulation of mitochondrial protein import (Weidberg & Amon 2018); the discovery of ATAD1 as a neuroprotective molecule expanded ATAD1's function to the regulation of synaptic activities (Dai et al. 2010, Zhang et al. 2011b). As the mechanistic details of these processes are lacking, however, how their mechanisms and the mechanism of TA protein extraction can be unified is yet unknown. Finally, it also remains to be understood what role Msp1/ATAD1 plays under different stress conditions and in different cell types, and how it is integrated into the complex cellular stress response network. To this end, defining the full proteome of Msp1/ATAD1's substrates under different cellular contexts will provide valuable insights.

## DISCLOSURE STATEMENT

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## Errata

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