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Quality control of mislocalized and orphan proteins

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ABSTRACT

A healthy and functional proteome is essential to cell physiology. However, this is constantly being challenged as most steps of protein metabolism are error-prone and changes in the physico-chemical environment can affect protein structure and function, thereby disrupting proteome homeostasis. Among a variety of potential mistakes, proteins can be targeted to incorrect compartments or subunits of protein complexes may fail to assemble properly with their partners, resulting in the formation of mislocalized and orphan proteins, respectively. Quality control systems are in place to handle these aberrant proteins, and to minimize their detrimental impact on cellular functions. Here, we discuss recent findings on quality control mechanisms handling mislocalized and orphan proteins. We highlight common principles involved in their recognition and summarize how accumulation of these aberrant molecules is associated with aging and disease.

1. The need for protein quality control

Proteins are involved in virtually every cellular process. To perform their roles, proteins need to be expressed at the right concentration and time, fold into their unique three-dimensional structures, be transported to the correct subcellular compartments, and assemble with other proteins into complexes. The maintenance of a functional proteome with proper abundance, folding, localization and assembly of all cellular proteins is referred to as proteome homeostasis or proteostasis [1]. Failure to achieve proteostasis may result in the accumulation of aberrant proteins, including mutated, misfolded, mislocalized, orphaned or damaged species. These aberrant proteins are often aggregation-prone and potentially toxic, posing a continuous burden to cells [2,3]. In fact, a decline in proteostasis is associated with aging, cancer, and some late-onset neurodegenerative diseases [4].

To maintain proteostasis, cells employ sophisticated protein quality control systems that operate in different organelles in order to handle aberrant proteins (reviewed in Refs. [2,3,5–10]). Faulty proteins can be degraded, for removal from the intracellular environment, or be rectified through disaggregation and refolding. Formation of protein aggregates is another strategy to minimize potential toxic effects of aberrant proteins by actively sequestering them into defined deposition sites [11–15]. These processes are mediated by dedicated quality control factors, including molecular chaperones for detecting aberrant proteins and determining their triage between attempts to regain the native structure, sequestration at deposition sites, or commitment to degradation, as well as the ubiquitin-proteasome and autophagy machineries for degrading proteins [2,3]. Protein targeting and translocation factors may also be considered part of the proteostasis network, as they effectively prevent mislocalization by shuttling nascent proteins to correct compartments. In this review, we focus on quality control systems that handle mislocalized and orphan proteins.

2. Quality control of mislocalized and orphan proteins

Mislocalized proteins are defined as proteins that fail to reach their native compartments, while orphan proteins are those that fail to assemble into native complexes (Fig. 1). Proteins destined to a defined organelle are recognized by the corresponding targeting machinery through signal sequences or structural motifs. However, due to similarities between signal sequences that target proteins to different compartments, mislocalized proteins can be generated inherently since it is possible for a particular protein to be recognized by the wrong targeting machinery [16–22] (Fig. 1a). The limited capacity of targeting pathways can also lead to some degree of protein mislocalization even under normal circumstances [22–24]. These aberrant behaviors are often exacerbated under stress conditions. For example, proteotoxic stress in the endoplasmic reticulum (ER) and mitochondria can suppress import of proteins into these organelles, causing their mislocalization to the cytosol [25–28]. In addition, mutations within signal sequences can

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cause protein mislocalization, as shown by the classical examples of several disease-related mutations in the nuclear proteins FUS and TDP-43. Mutations in these proteins can lead to their cytoplasmic mislocalization, and ultimately the onset of amyotrophic lateral sclerosis [29–31].

Like mislocalized proteins, orphan proteins can also be produced inherently as expression of protein complex subunits is not perfectly stoichiometric, resulting in some excess subunits that cannot assemble into complexes [32–34] (Fig. 1b). Although nature has evolved means to facilitate and control assembly of complexes [35,36], pervasive loss of stoichiometry of complex subunits is observed during aging [37–39]. Finally, as subunits of many protein complexes are encoded by genes located on different chromosomes, changes in chromosome copy number, as seen in aneuploidy, can also lead to unbalanced synthesis of complex subunits, and therefore orphan proteins [40,41].

Similar to other aberrant proteins, accumulation of mislocalized and orphan proteins can cause proteotoxicity. Both mislocalized and orphan proteins may misfold due to changes in folding environment (such as pH) or lack of binding partners [42–46]. This can lead to exposure of hydrophobic surfaces that would normally be buried within the properly folded conformation. Even without misfolding, some mislocalized membrane proteins can expose transmembrane domains due to their failure to insert into membrane structures. The same can happen to interaction interfaces of orphan proteins due to absence of binding partners. These abnormal features can induce proteotoxicity through various mechanisms, including promiscuous interactions with important cellular proteins such as protein quality control and nucleocytoplasmic transport factors, or damage to the integrity of membrane structures [47–51].

How can quality control systems detect mislocalized and orphan proteins but not their properly localized and assembled counterparts? It appears that the above-mentioned abnormal features causing proteotoxicity can be recognized by quality control systems (Fig. 1). Given that mislocalized proteins can also be orphan due to absence of their binding partners in the compartments to which they are wrongly localized, some mislocalized proteins may be recognized by the same mechanisms as orphan proteins. Supporting the notion that such recognition is highly specific, proteins exhibiting dual localization are not recognized as mislocalized and do not elicit a quality control response. For example, the C-terminus of the human tail-anchored protein Fis1 interacts with the peroxisomal protein Pex19, which promotes its insertion into the peroxisomal membrane. However, if this interaction is precluded, the same C-terminal sequences can direct Fis1 to the mitochondrial outer membrane, where it is involved in mitochondrial fission [52]. Other examples of dual-localized proteins include the stress-activated transcription factor ATFS-1, which contains both nuclear and mitochondrial signal sequences, the NADH-cytochrome b(5) reductase and cytochrome P450 monooxygenases, which localize to both ER and mitochondria [53–55].

Work over the last decades has shown that eukaryotic cells have evolved quality control pathways to handle mislocalized and orphan proteins through degradation or spatial sequestration, so as to minimize their toxic effects. In principle, such quality control pathways should exist in every subcellular compartment as emergence of mislocalized and orphan proteins is expected to be ubiquitous. In the following sections, we discuss recent discoveries regarding detection and degradation of mislocalized and orphan proteins in different compartments ranging from the cytosol, mitochondria and ER, to the inner nuclear membrane. We highlight emerging evidence suggesting that quality control of mislocalized and orphan proteins follow similar principles. In addition, we discuss recent studies proposing spatial sequestration as an alternative way to manage mislocalized and orphan proteins. Finally, we explore links between accumulation of these aberrant proteins and various diseases.

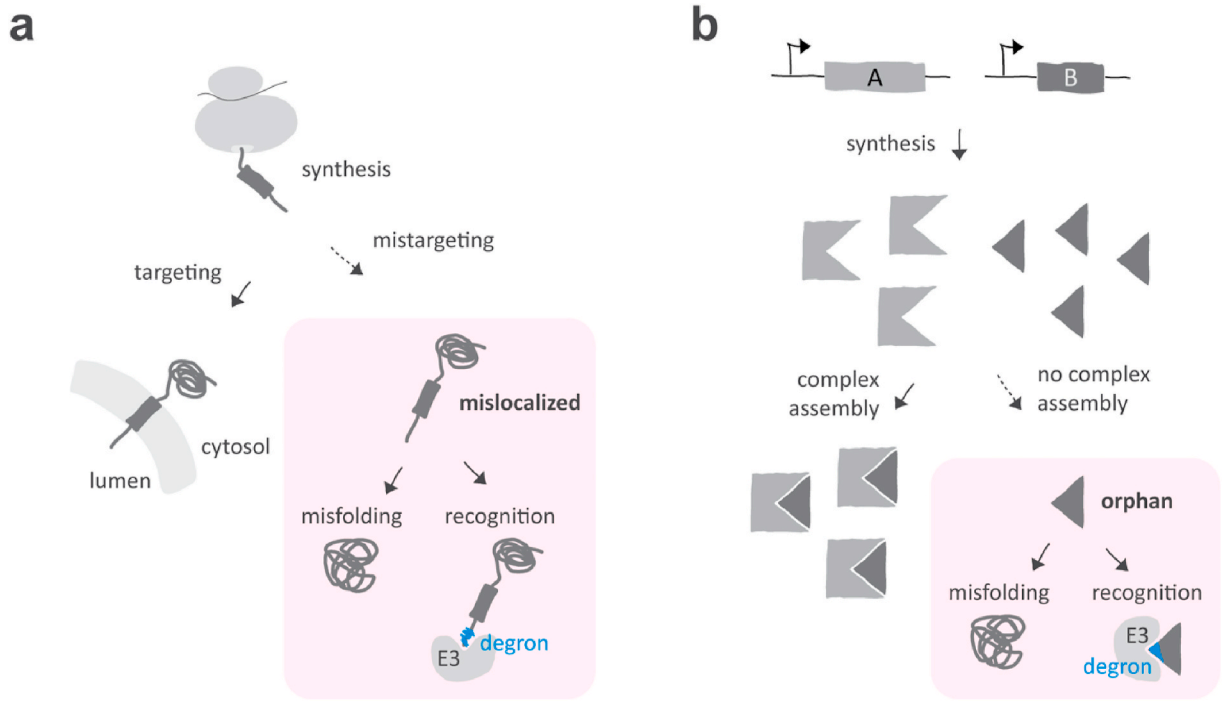

Fig. 1. Mislocalized and orphan proteins. **a** – Protein targeting is error-prone and can lead to protein mislocalization. In this example a hypothetical membrane protein is mistargeted, localizing to the cytosol (pink square). There it can either misfold or be recognized, directly or indirectly, by a ubiquitin-protein ligase (E3) and marked for proteasomal degradation. Recognition is achieved via a degron that is exposed in the mislocalized species but is not accessible in the correctly localized molecules. **b** – The process of protein complex assembly can produce aberrant molecules. In this example of a hypothetical complex composed of two different subunits, one subunit is orphaned due to lack of binding partners (pink square). An orphan subunit can either misfold or be recognized and marked for destruction by a ubiquitin-protein ligase (E3) via an exposed degron, which is otherwise concealed in the correctly assembled complexes.

3. Degradation as the first line of defense

Most mislocalized and orphan proteins are targeted for selective degradation by the ubiquitin-proteasome system. Selective degradation of these aberrant proteins appears to involve a common principle: exposure of some structural features or sequence motifs, known as degradation signals or degrons, that are recognized by substrate-specific ubiquitin-protein ligases (E3 ligases hereafter), followed by substrate ubiquitylation and proteasomal degradation (Fig. 1). These degrons can be classified into two types, depending on whether the protein is misfolded or not. The first type involves hydrophobic regions that are typically buried within the functional protein fold but become exposed upon protein misfolding. The second type involves features that become exposed independently of misfolding, e.g., transmembrane domains in membrane proteins mislocalized to the cytosol or interfaces involved in interactions between complex subunits in orphan proteins. Mislocalized and orphan proteins that misfold appear to be targeted for degradation by the same pathways that recognize other types of misfolded proteins, while those with degrons of the second type are often handled by dedicated quality control factors. Within this general principle, the exact degrons and the involved E3 ligases vary between different mislocalized and orphan proteins, as we describe in the following sections.

3.1. Proteins mislocalized to the cytosol

Proteins that are destined to different organelles may remain in the cytosol due to inefficient targeting, either because of failure in their targeting machineries, or mutations within their signal sequences. Although some of these mislocalized proteins are eventually imported and degraded in the nucleus, here we consider them as mislocalized to the cytosol as it is where they are first detected by the quality control machinery. Consistent with the general principle described above, some of these mislocalized proteins may become misfolded. For these misfolded species, molecular chaperones can bind to their exposed hydrophobic surfaces and recruit specific E3 ligases for their ubiquitylation, which marks them for subsequent proteasomal degradation. For instance, in the case of the yeast carboxypeptidase Y and the vacuolar proteinase A, removal of their ER signal sequences results in mutant proteins that mislocalize to and misfold in the cytosol [45,46]. Recognition of these mutant proteins involves the chaperones Ssa1 and Ydj1, which are heat shock proteins of the Hsp70 and Hsp40 families [56], whereas their subsequent ubiquitylation requires the cytosolic E3 ligase Ubr1 [45,57–59]. Alternatively, these mislocalized proteins can be imported into the nucleus through the combined action of the chaperones Ssa1, Ydj1, Sse1, and Sis1 [45,50,57], and then ubiquitylated by the nuclear E3 ligase San1 [45,57–59].

A prominent group of mislocalized proteins that likely are not recognized due to misfolding is membrane proteins. If not properly inserted into membrane structures, these proteins can accumulate in the cytosol and thus expose their transmembrane domains. In mammalian cells, these transmembrane domains can be recognized by the ribosome-associating chaperone Bag6, or by ubiquilins, a family of proteins that recruit poly-ubiquitylated proteins to the proteasome [60]. The Bag6 pathway was first revealed using the mammalian prion protein PrP *in vitro* [61]. Interestingly, before functioning as a degradation factor by directing its clients for ubiquitylation by the RNF126 E3 ligase [62], Bag6 first facilitates their recruitment to targeting factors, such as TRC40 in the case of tail-anchored (TA) proteins (with a single transmembrane domain at the C-terminus), for insertion into the ER [63]. Thus, Bag6 is a triage factor that promotes both productive targeting and rapid degradation if translocation fails.

The ubiquilin pathway is mediated by the four main ubiquilins (UBQLN1-4) in human cells, among which UBQLN1, 2, and 4 are ubiquitously expressed [60]. Originally shown to target the mutant membrane protein IL-2Rα ΔSS to proteasomal degradation, as it mislocalizes to the cytosol due to lack of its ER signal sequence (SS), this pathway is also involved in the degradation of endogenous unimported ER and mitochondrial proteins [64–66]. Given that UBQLN4 is a Bag6-interactor, the Bag6 and ubiquilin pathways are not completely independent and may cooperate in quality control of mislocalized proteins, as shown for IL-2Rα ΔSS [66]. In contrast to Bag6, however, the role of ubiquilins with respect to substrate ubiquitylation is not clear. In the case of IL-2Rα ΔSS, the amount of ubiquitylated IL-2Rα ΔSS species increases upon UBQLN4 knockdown but decreases upon Bag6 knockdown, suggesting that UBQLN4 functions after, while Bag6 functions before, ubiquitylation [66]. For the mislocalized mitochondrial protein Omp25, an unknown E3 ligase can be co-purified with an *in vitro*-synthesized complex containing Omp25 and UBQLN1, suggesting a direct role of UBQLN1 in E3 ligase recruitment [65]. It remains to be determined whether Bag6 is also part of this complex, and whether UBQLN1 and UBQLN4 are functionally distinct. Despite the identification of the Bag6 and ubiquilin pathways, more quality control factors for proteins mislocalized to the cytosol likely remain to be found. Supporting this notion, different unimported mitochondrial intermembrane space proteins in yeast are targeted by a wide repertoire of substrate-specific ubiquitylation factors, and no single E3 ligase appears to be involved in the degradation of all of them [67].

3.2. Orphan proteins in the cytosol

Orphan proteins may be produced in the cytosol as a result of unbalanced synthesis of complex subunits or due to mislocalization of proteins which have their binding partners in a different compartment. Similar to mislocalized proteins, these orphan proteins may misfold. One such example is the human tumor suppressor von Hippel-Lindau (VHL) exogenously expressed in yeast, which becomes orphan and misfolds in the cytosol due to absence of its binding partners elongins B and C [68,69]. Interestingly, VHL is modified with K48- and K11-linked poly-ubiquitin chains (reviewed in Refs. [70,71]) by distinct circuitries, each involving a different set of chaperones and E3 ligases [69]. Both K48 and K11 ubiquitylation linkage types are necessary for degradation, thus providing a double checkpoint mechanism that prevents unwanted degradation of properly assembled and folded proteins.

On the other hand, orphan proteins with no apparent misfolding often expose interaction surfaces that are normally shielded by their complex binding partners. Several examples indicate that these surfaces can be detected by a variety of factors, including Tom1/HUWE1, UBE2O, Not4, Teb4, UBR1 and related E3 ligases.

HUWE1 is a HECT (homologous to the E6-AP carboxyl terminus) domain E3 ligase. Together with the ubiquitin-selective chaperone Cdc48/p97, HUWE1 ubiquitylates orphan Ubl4A, which is a member of the chaperone holdase complex that also contains Bag6 and TRC35 [72]. A hydrophobic segment within Ubl4A that is normally shielded by Bag6 appears to be the degron that initiates HUWE1-mediated ubiquitylation [72]. In addition to Ubl4A, HUWE1 can also recognize subunits of many nuclear complexes, e.g., the splicing factor SF3B6 [72]. More recently, the E2/E3 hybrid ubiquitin-protein ligase UBE2O was implicated in targeting for proteasomal degradation the GFP-tagged ribosomal protein RPL24, which cannot assemble with other ribosomal subunits due to steric hindrance [73]. However, UBE2O can only modify its substrates by mono-ubiquitylation, and it is not known whether an E3 ligase is involved in extending mono-ubiquitin to poly-ubiquitin chains [73]. It is conceivable that both HUWE1 and UBE2O pathways target orphan subunits of nuclear protein complexes when they are mislocalized to the cytosol, as ubiquitylation and degradation of SF3B6 and GFP-RPL24 can be enhanced by blocking their nuclear import [72,73]. These examples also highlight the overlap between orphan and mislocalized proteins. Orphan ribosomal subunits can also be targeted for degradation by HUWE1, or in yeast by its homolog Tom1 [74]. In yeast, degradation of orphan ribosomal subunits appears to occur in the nucleus, as Tom1 is a nuclear protein, and they accumulate in the nuclear fraction upon Tom1 inactivation [74]. However, their degradation site in mammalian cells is

not clear, as HUWE1 was shown to be in the nucleus [75,76], in the cytosol [72], or to translocate from the cytosol into the nucleus upon DNA damage [77,78]. Further work is required to understand whether selective degradation of orphan ribosomal subunits by HUWE1, Tom1, and UBE2O constitute the same pathway or are independent from one another. Tom1 appears to recognize orphan ribosomal subunits through some accessible positively charged residues that are normally part of interaction interfaces in assembled ribosomes [74]. This indicates that the degrons triggering quality control may not necessarily be hydrophobic sequences and, together with the Ubl4A example, suggests the ability of interaction interfaces to act as degrons. Although these regions are primarily recognized by ubiquitylation factors, in some cases chaperones may be required to maintain orphan proteins in a soluble, degradation-competent state, as shown for the Fas2 subunit of the yeast fatty acid synthase complex in the absence of its partner Fas1 [79].

In several cases, recognition of orphan proteins appears to involve their acetylated N-termini, also known as Ac/N-degrons, which can direct proteins towards degradation via the Ac/N-degron pathway (reviewed in Refs. [80,81]). Generation of such Ac/N-termini occurs co-translationally and is mediated by N-terminal acetyltransferases according to the residue after the initiator methionine [80,82,83]. At least for some protein complex subunits, Ac/N-termini appear to be located at interaction interfaces, and therefore can only become accessible when the subunits are orphaned, resulting in their ubiquitylation and proteasomal degradation. One example is the Cog1 subunit of the yeast oligomeric Golgi complex, which also contains the Cog2-8 subunits. Overexpressed Cog1, whose N-terminus is acetylated by the N-terminal acetyltransferase NatB, fails to assemble into native oligomeric Golgi complexes. Such orphaned Cog1 is degraded through the Not4 E3 ligase in a NatB-dependent manner [84]. Its stabilization upon simultaneous overexpression of Cog2, Cog3, and Cog4 indicates that only orphan Cog1 is targeted for degradation [84]. In mammalian cells, the G protein regulator Rgs2 is regulated by an analogous mechanism. The N-terminus of Rgs2 is also acetylated by NatB. Consistently, degradation of overexpressed and thus orphan Rgs2 depends on NatB as well as the Teb4 E3 ligase (homolog of yeast Doa10) and is inhibited by co-expression of the Rgs2 partner Gαq [85]. Similarly, in the absence of the FNTB subunit of the farnesyl transferase complex, the other subunit FNTA seems to be degraded via an N-degron pathway through the UBR1, UBR2, and UBR3 E3 ligases [72], although the possible involvement of an Ac/N-degron has not been tested. Intriguingly, N-terminal acetylation can in some cases promote protein complex assembly [86]. It is thus possible that Ac/N-termini function analogously to a triage factor in both complex assembly and degradation of orphan subunits upon assembly failure. Considering that most eukaryotic proteins (>50% in yeast and >80% in human) are acetylated at their N-termini [80,82,83], recognition of orphan proteins through exposed Ac/N-termini is likely not limited to the above-mentioned examples. More work is required to determine the prevalence of Ac/N-termini as degrons in orphan proteins, given that most N-termini cannot function as degrons when fused to a reporter protein and loss of N-terminal acetyltransferases has little impact on proteostasis [87,88].

The pathways described thus far cope with orphan proteins that are expected to be monomeric. A different quality control mechanism that targets aberrant dimers of BTB domain-containing proteins was recently described [89]. In contrast to functional homodimers formed by two identical BTB proteins, these aberrant dimers are either mutant homodimers or heterodimers that emerge when a BTB protein pairs up with its mutated counterpart or a different BTB protein, respectively. The E3 ligase SCF^{FBXL17}, which contains SKP1, CUL1, and the F-box protein FBXL17, recognizes and ubiquitylates these aberrant dimers, thereby targeting them to proteasomal degradation in a process that was termed dimerization quality control [89]. SCF^{FBXL17} recognizes aberrant dimers via residues that are only exposed upon aberrant dimerization, and/or via a displaced N-terminal β-strand that is involved in the formation of an intermolecular β-sheet in BTB homodimers [89,90]. Therefore, SCF^{FBXL17} can detect the complementarity between two BTB proteins within a dimer, which enables it to function as a quality control factor for ~200 human BTB proteins. It will be interesting to determine whether similar factors exist that target aberrant dimers formed by proteins containing other common dimerization domains, such as leucine zippers and coiled coils.

3.3. Tail-anchored proteins mislocalized to mitochondria

TA proteins are membrane proteins that contain a single transmembrane domain at their C-terminus. This location of the transmembrane domain precludes the use of ER co-translational targeting pathways like the signal recognition particle [91]. Instead, TA proteins are recognized via their transmembrane domain and inserted into the ER membrane post-translationally by the GET (Guided Entry of Tail-anchored proteins)/TRC (Transmembrane domain Recognition Complex) pathway [16,92,93] or the conserved EMC (ER Membrane protein Complex) pathway [94,95]. TA proteins can also localize to other compartments, including the peroxisomal membrane, mainly via trafficking through the ER, or the mitochondrial outer membrane. Notably, due to limited fidelity of their targeting pathways, some TA proteins may end up in the wrong membrane. One such example is the yeast peroxisomal TA protein Pex15, which can be mistargeted to the mitochondrial outer membrane even under physiological conditions [96] (Fig. 2). Exacerbating Pex15 mistargeting by either deletion of its peroxisomal signal sequence to create Pex15Δ30, or by deletion of GET components, provides a robust system for investigating mechanisms of its quality control. This led to the identification of Msp1 and its mammalian homolog ATAD1 as key quality control factors [96,97]. Msp1 is a AAA-ATPase anchored to both mitochondrial outer membrane and peroxisomal membrane via its N-terminal transmembrane domain, with its C-terminal ATPase domain in the cytosol [98]. It functions as a ring-shaped hexamer and uses its ATPase activity to unfold and extract substrate proteins from membranes through its central pore [99,100]. Using Pex15Δ30 as a model, it was shown that proteins extracted by Msp1 can be degraded by the cytosolic proteasome after their ubiquitylation by the E3 ligase Doa10 working together with the Ubc6/7 ubiquitin-conjugating enzymes [101]. Alternatively, extracted Msp1 substrates can reinsert into the ER membrane, where they are ubiquitylated by Ubc6/7 and Doa10, and finally extracted by another AAA-ATPase Cdc48, followed by proteasomal degradation [102] (Fig. 2). This ER-reinsertion mechanism could provide an opportunity to reroute ER-destined TA proteins back to their correct localization.

Interestingly, Pex15 correctly inserted into the peroxisomal membrane is protected from targeting by Msp1, in part because Msp1 is inhibited by another peroxisomal protein Pex3 [99]. Another way of protection is provided by the direct association between Pex15 and Pex3, which possibly shields two features important for Pex15 recognition by Msp1: a hydrophobic patch in the Pex15 cytosolic domain and some positively charged residues in its C-terminal tail [103,104] (Fig. 2). Pex15 mislocalized to the mitochondrial outer membrane is orphaned by lack of Pex3 and therefore can be recognized by Msp1, highlighting the overlap between mislocalized and orphan proteins. Beyond its role in quality control of TA proteins, Msp1 is also involved in clearance of mitochondrial import intermediates from clogged TOM (Translocase of the Outer Membrane) complexes [105,106]. This activity involves stress-induced expression of the Cis1 adaptor, which recruits Msp1 to the TOM complex [106,107]. It is possible that as yet unidentified Cis1-like adaptors also play a role in Msp1-dependent quality control of proteins mislocalized to the mitochondrial outer membrane.

3.4. Mislocalized and orphan proteins in the ER and inner nuclear membrane

Similar to other compartments, mislocalized and orphan proteins in the ER can also be recognized and targeted for degradation. Indeed, the

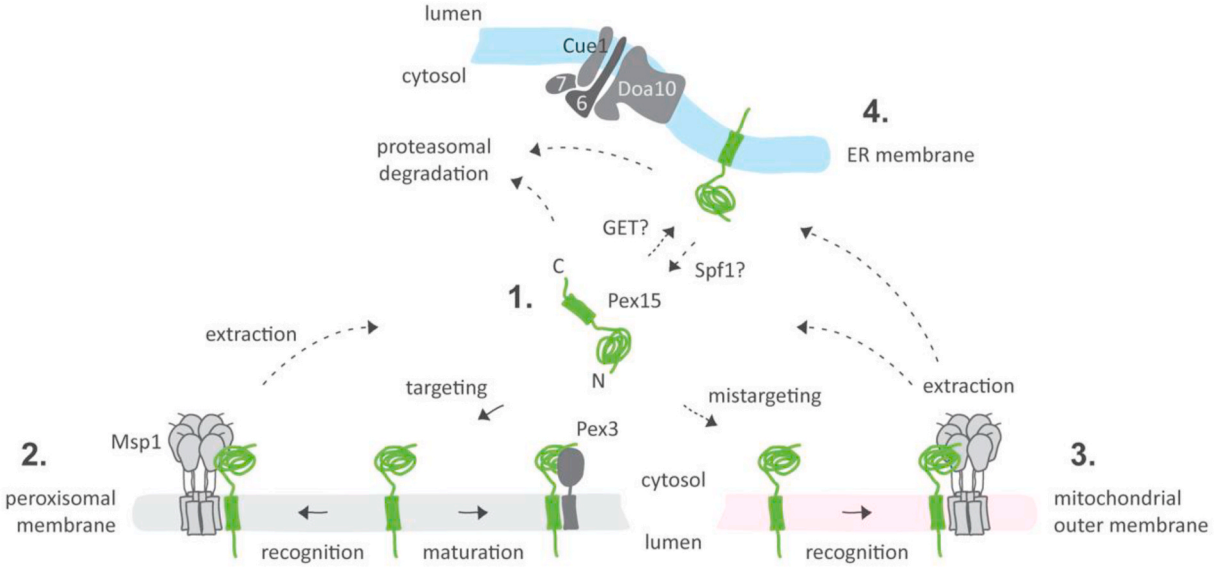

Fig. 2. Model of quality control of the yeast tail-anchored protein Pex15. Pex15 (1) is normally inserted into peroxisomes (2). Here it matures by interacting with the peroxisomal membrane protein Pex3 [104], and excess Pex15 can be extracted by the Msp1 AAA-ATPase. Pex15 can also erroneously insert into the mitochondrial outer membrane [96] (3), from where it is extracted by Msp1 either into the cytosol [99,100] or extracted and inserted into the ER membrane [102] (4). Cytosolic Pex15 or Pex15 located in the ER can be targeted for proteasomal degradation by the Doa10 ubiquitin-protein ligase with the Ubc6 and Ubc7 ubiquitin-conjugating enzymes [101,102]. The GET pathway and the Spf1 P5A-ATPase have been implicated in Pex15 turnover but their roles are not well defined [101].

ER was the first organelle where targeted recognition and degradation of orphan proteins were discovered. It was found that the ER chaperone BiP, a member of the Hsp70 family, binds specifically to unassembled immunoglobulin heavy chains [108,109]. Although initially the role of BiP was unclear, it was later discovered that its binding to the heavy chains persists until they meet their partner light chains, or otherwise commits them to degradation [110,111]. In agreement with the general role of BiP in the ER quality control processes [112], it was found to also bind to unassembled T cell receptor (TCR) α-chains [113,114]. In this case, however, the underlying mechanism turned out to be quite unexpected. Unassembled TCR α-chains have unusually polar transmembrane regions that are critical for TCR complex assembly and transport [115,116]. If TCR α-chains do not assemble properly in the ER membrane, they enter the lumen where BiP can bind to their transmembrane regions and target them to degradation through the E3 ligase Hrd1 [114,117–119]. It is the low hydrophobicity of the transmembrane region that leads to unstable membrane integration of the TCR α-chains and mislocalization into the ER lumen when they cannot assemble with their membrane-embedded partners. In contrast to previously detailed examples of mislocalized proteins recognized due to their orphan status, the case of TCR α-chains shows how an orphan protein may be detected based on its concomitant mislocalization.

An alternative mechanism to handle orphan TCR α-chains, and also pre-TCR α-chains (a subunit of the pre-TCR) [120], is mediated by the intramembrane serine rhomboid protease RHBDL4 [121]. For both proteins, positive charges in their transmembrane segments are usually masked by the binding of TCR β-chains [114]. If this interaction fails, the orphan α-chains are subjected to ubiquitylation by the E3 ligase gp78, followed by their intramembrane cleavage by RHBDL4, and finally extraction from the ER membrane by Cdc48/p97 for subsequent proteasomal degradation in the cytosol [121,122]. Another component of the TCR complex is the CD3-δ subunit. In the absence of its cognate TCR interaction partners, CD3-δ is degraded after being ubiquitylated by gp78 and another E3 ligase TRIM13/RFP2 [123–125]. However, the molecular features involved in orphan CD3-δ recognition remain ill-characterized.

Unassembled TCR α-chains and CD3-δ are model substrates of ER-associated degradation (ERAD). ERAD is a set of well-characterized and conserved pathways for degrading mainly misfolded or faulty ER proteins. Its substrates are typically recognized by ER chaperones such as BiP, probably through their exposed transmembrane or other hydrophobic regions, then ubiquitylated by the Doa10 or Hrd1 E3 ligases in yeast, and extracted from the ER by Cdc48 for proteasomal degradation (reviewed in Ref. [126]). ERAD can also target mislocalized and orphan proteins in the ER [10,42], such as the overexpressed Stt3 subunit of the yeast oligosaccharyl transferase complex, and the orphan subunits of the human epithelial sodium channel [127–132]. The CAML subunit of the mammalian ER insertase involved in the TRC pathway is also destabilized upon depletion of its partner WRB [133]. Orphaned CAML misfolds and exposes one of its transmembrane domains, although whether its degradation is mediated through ERAD remains to be determined. It is likely that other pathways that detect orphan ER proteins independently of misfolding also exist. For instance, orphan WRB is rapidly degraded even though it remains correctly folded in the absence of CAML [133]. It will be interesting to understand how orphan but correctly folded WRB is recognized as aberrant.

The ER membrane is continuous with the outer nuclear membrane, which is connected to the inner nuclear membrane via nuclear pores. Thus, some membrane proteins can diffuse from the ER to the inner nuclear membrane [134]. Protein composition within the inner nuclear membrane appears to be maintained by the yeast Asi E3 ligase complex, which contains the Asi1 and Asi3 subunits for substrate ubiquitylation. The Asi complex cooperates with Ubc6/7 and Cdc48 in targeting for degradation proteins mislocalized to the inner nuclear membrane, such as the ER membrane protein Erg11 involved in sterol biosynthesis [135,136]. The vacuolar proteins Vtc1 and Vtc4, which mislocalize to the inner nuclear membrane upon C-terminal tagging, and a mutant of the ER translocon subunit Sec61, which is forced to the inner nuclear membrane by fusion to a nuclear localization signal, are also degraded in an Asi-dependent manner [135,136]. Interestingly, Asi1 can ubiquitylate the nucleoporin Pom33 and thereby ensure its correct uniform distribution along the nuclear envelope [137]. This provides another mechanism through which the Asi complex may regulate protein content and distribution at the inner nuclear membrane. Furthermore, orphan subunits of some ER complexes are degraded after their diffusion to the inner nuclear membrane. This has been shown for Wbp1, another subunit of the oligosaccharyl transferase complex [138]. Together with the TCR α-chains that assemble in the ER membrane but are recognized

in the lumen, the quality control mechanism of orphan Wbp1 suggests that spatial segregation of assembly and degradation may be exploited for providing more time and space for a complex subunit to find its binding partners. Wbp1 and Erg11 are recognized through their transmembrane domains by Asi2, another Asi complex subunit [138]. It remains to be determined what features within other Asi substrates are necessary for their recognition by this complex.

In an opposite way to Pex15, mitochondrial membrane proteins may also be mistargeted to the ER membrane. A recently identified mechanism for their quality control involves extraction from the ER membrane into the cytosol, presumably for proteasomal degradation or another chance at correct mitochondrial insertion [139]. This mechanism requires the ER-resident P5A-ATPase transporter Spf1 (in yeast) or ATP13A1 (in human). Spf1/ATP13A1 preferentially recognizes mistargeted mitochondrial membrane proteins that contain transmembrane domain with some positively charged sequences on either side, and then mediates their extraction through ATPase-dependent conformational switching [139]. Therefore, it acts analogously to mitochondrial Msp1 in safeguarding the composition of membrane proteins at their respective organelles. Similar functions have also been proposed for CATP-8, the *C. elegans* homolog of Spf1/ATP13A1 [140].

Mitochondrial membrane proteins mistargeted to the ER can also be directly rerouted to mitochondria in a pathway dependent on the ER-localized chaperone Djp1. In yeast, mitochondrial proteins such as the inner membrane protein Oxa1 are maintained in an import-competent state by Djp1 when they are being mistargeted to the ER and can then be redirected to mitochondrial import [141]. A significant portion of mitochondrial proteins appears to reach mitochondria via this pathway, as Djp1 deletion affects their proper import in cells with an otherwise intact mitochondrial translocation machinery [141]. Together with the observation that some ER-destined TA proteins mistargeted to the mitochondria may also be re-translocated to the ER [102], it seems likely that there is constant protein exchange between the ER and mitochondria that serves as a degradation-independent mechanism to reduce or correct protein mislocalization.

4. Protein aggregation as an alternative quality control strategy

Formation of insoluble protein aggregates is a hallmark of many neurodegenerative diseases [142]. Hence, it was traditionally assumed that protein aggregation is toxic to cells [143]. However, a growing body of evidence suggests that the smaller protein oligomers (intermediates of the aggregation process) are the major toxic species [144–146], and that protein aggregation may even be beneficial to cell survival [14]. In fact, formation of some protein aggregates appears to be an active and organized process. The concept of organized protein aggregation was first established by a study in yeast that examined the fate of orphan, and therefore misfolded, VHL upon proteasomal inhibition [11]. This revealed the presence of two defined deposition sites for VHL: the juxta-nuclear quality control compartment (JUNQ), where VHL preferentially aggregates, and the peri-vacuolar insoluble protein deposit (IPOD), where VHL can aggregate upon additional proteotoxic stress such as heat shock. Subsequent analyses indicated that JUNQ is in fact an intra-nuclear quality control compartment (INQ) [12]. Deposition of VHL into INQ/JUNQ and IPOD are active processes that require the small heat shock proteins Btn2 and Hsp42, respectively [12,147]. These types of aggregation provide a way to remove unwanted proteins from a dividing cell population through their asymmetric partitioning during cell division [148]. Moreover, aggregation can also serve to prevent overload of the proteasome by sequestrating VHL and probably other orphan or misfolded proteins in a state that is competent for degradation or refolding during recovery from stress [149,150].

In addition to VHL, orphan proteasomal or ribosomal subunits can also aggregate. In yeast, a C-terminal truncation mutant of the proteasome lid subunit Rpn5 (Rpn5ΔC), which causes proteasome misassembly, co-aggregates with another lid subunit Rpn11 at the IPOD in a Hsp42-dependent manner [151]. Some unassembled ribosomal subunits also shift to the insoluble protein fraction upon chemical inhibition of ribosomal assembly or in the absence of the Tom1 E3 ligase [74,152]. How these aggregates are related to INQ/JUNQ and IPOD has not been investigated in detail. In mammalian cells, heat shock can induce HUWE1-mediated modification of the ribosomal subunit RPL7 with hybrid neddylation (attachment of the ubiquitin-like protein NEDD8) and ubiquitylation chains, which promote its transient aggregation and thereby prevent proteasome overload [76]. The shift of HUWE1 activity from sole ubiquitylation to mixed neddylation and ubiquitylation upon heat shock seems to depend on the activation of NEDD8 by the E1 enzyme UBA1 [76]. Interestingly, aggregation may not be only confined to subunits that are completely incompetent for complex assembly but appears to compete with the assembly process. This is supported by the observation that Hsp42 knockout leads to assembly of partially functional proteasomes containing Rpn5ΔC [151]. Thus aggregation may be a safety mechanism to temporarily sequester excess complex subunits that cannot be promptly assembled.

The importance of aggregation as a quality control strategy is further supported by a quantitative proteomics study of aggregated proteins in aneuploid yeast strains with an extra copy of different chromosomes [153]. This analysis demonstrated that protein aggregation is an effective way to reduce the amount of overexpressed complex subunits in the soluble pool. How excess subunits are selected for aggregation, the location of their aggregates and the aggregation factors involved remain to be elucidated. Moreover, widespread aggregation of proteins that exceed their solubility threshold can be observed in aging *C. elegans*, which is associated with stoichiometry loss of some protein complex subunits [37]. Taken together, aggregation is an integral part of quality control pathways for orphan proteins that affects a wider range of substrates than initially assumed. Besides aggregation, stress-induced phase separation was recently proposed as another way to concentrate unassembled ribosomal subunits with proteasomes, likely for their more efficient degradation, and to temporarily store aberrant proteins, thus minimizing their toxic effects [154,155].

5. Mislocalized and orphan proteins in aging and disease

Aging and some human diseases, including neurodegeneration and cancer, are associated with accumulation of mislocalized and orphan proteins, or defects in their quality control. First, aging may lead to pervasive protein mislocalization due to deterioration of nuclear pore complexes and reduced efficiency of mitochondrial import [156,157]. This is also supported by the observation that localization of some protein kinases and transport factors is altered in aged animal models [38]. Furthermore, aging is correlated with stoichiometry loss of multiple protein complexes, including the ribosome, proteasome, and nuclear pore, at least partially due to deregulation of translation machinery [37–39].

Amyloid-like aggregates associated with neurodegenerative diseases have the ability to disrupt nucleocytoplasmic protein transport by sequestrating nuclear importins and thereby causing their cytosolic mislocalization [51]. In addition, mitochondrial import is inhibited in various experimental models of Alzheimer's, Parkinson's and Huntington's diseases [158–160]. Expression of UBQLN1, which is involved in degradation of some mislocalized proteins in the cytosol, appears to be limiting in Alzheimer's and Huntington's, as its overexpression can alleviate cytotoxicity and disease symptoms [161,162]. All these mechanisms increase the load of mislocalized and orphan proteins in aging or neurodegeneration, thus reducing, or together with other aberrant proteins, even saturating the capacity of protein quality control systems. This may then further impair their quality control and exacerbate their accumulation, resulting in a vicious cycle that eventually leads to proteostasis collapse and cell death.

Mislocalized and orphan proteins are also pervasive in cancers, particularly in those associated with aneuploidy as found in most solid

tumors [41,163,164]. Although the effect of imbalances in gene copy number is somewhat buffered post-transcriptionally, especially for subunits of complexes [165,166], aneuploid cancer cells still face a high burden of orphan proteins. Tumorigenesis is also frequently associated with protein mislocalization, as observed for many proteins with oncogenic, tumor suppressive, or other functions [167]. Together with the induction of protein misfolding due to the stressful surrounding microenvironment, cancer cells are subjected to constant challenges to their proteome integrity, and therefore show increased dependency on protein folding and quality control systems to sustain their rapid growth. Consistently, several inhibitors of the Hsp90 chaperone are being actively tested for their anti-cancer efficacy with some promising results [168–170]. Further understanding of the mechanisms and quality control factors that handle mislocalized and orphan proteins could therefore help identify novel targets and strategies for anti-cancer therapy.

6. Conclusions

Recent work using model substrates or genome-wide approaches has shown that eukaryotic cells are equipped with multiple and often conserved quality control pathways for handling a wide repertoire of mislocalized and orphan proteins. These pathways potentially exist in all subcellular compartments, with those functioning in the cytosol, mitochondria, ER, and inner nuclear membrane being best understood. Proteasomal degradation appears to be the preferred strategy to deal with mislocalized and orphan proteins, as this can completely remove these aberrant molecules from the intracellular environment, while aggregation can prevent them from disrupting functions of other proteins, sequestering chaperones or other protein quality control factors, and overloading the proteasome. Several examples suggest that mislocalized and orphan proteins may be detected and handled by similar mechanisms. As mislocalized and orphan proteins are highly diverse, in terms of both sources and localizations, it is likely that further factors, pathways, and strategies for their quality control remain to be identified. Given that orphan VHL is targeted for degradation by dual ubiquitylation linkage types, it will be interesting to determine whether and how different linkage types are involved in marking other mislocalized and orphan proteins. Another aspect to consider is based on the observation that the length of transmembrane domains varies between membrane proteins residing in different compartments [171–174]. It is thus tempting to speculate that this feature could be employed by cells to detect mislocalized membrane proteins. Considering that accumulation of mislocalized and orphan proteins is associated with aging and disease states, better understanding of their quality control may enable the design of novel therapeutic approaches.

Credit statement

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