

Review

Target Selection during Protein Quality Control

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Protein quality control (QC) pathways survey the cellular proteome to selectively recognize and degrade faulty proteins whose accumulation can lead to various diseases. Recognition of the occasional aberrant protein among an abundant sea of similar normal counterparts poses a considerable challenge to the cell. Solving this problem requires protein QC machinery to assay multiple molecular criteria within a spatial and temporal context. Although each QC pathway has unique criteria and mechanisms for distinguishing right from wrong, they appear to share several general concepts. We discuss principles of high-fidelity target recognition, the decisive event of all protein QC pathways, to guide future work in this area.

The Importance of Protein QC

QC pathways constantly monitor the proteins within cells for occasional errors or damage. To maintain homeostasis, aberrant proteins must be rapidly recognized and either corrected or degraded. Protein QC occurs in all cellular compartments and is required to detect a wide range of defects, including protein misfolding, mislocalization, and numerous types of damage [1]. Failure to recognize QC targets results in the accumulation of faulty products that may aggregate or have dominant negative consequences. By contrast, overly promiscuous recognition may erroneously target functional proteins for degradation, depleting the cell of resources. Defects in protein QC are linked to numerous diseases, most notably neurodegeneration (Box 1) [2,3]. Thus, understanding the mechanisms underlying protein QC pathways is an important goal in cell biology and may aid the development of therapeutics for QC-related diseases.

QC can be conceptualized as the process of surveying proteins relative to a prescribed definition of 'normal', and selectively committing for degradation polypeptides that fail to meet these criteria. Together, these steps comprise the decisive process of target selection. The subsequent steps of polypeptide degradation have been reviewed elsewhere [4–6]. It is impractical to comprehensively discuss all of the QC pathways distributed among the various compartments of eukaryotic cells. We therefore focus on general principles of target selection during protein QC illustrated by four case studies: **ribosome-associated quality control (RQC)**, QC of **mis-localized proteins**, glycoprotein QC, and membrane protein degradation.

The Challenges Facing Accurate Target Selection

Target selection during QC is extremely challenging for three major reasons. First, cells contain a diverse proteome with nearly all biochemical features being represented, such that the definition of 'normal' is exceptionally broad. At the same time, the range of defects any given protein may experience is essentially limitless: it can be misfolded to various degrees, modified inappropriately, mutated, or damaged in numerous ways. Hence, defects in one protein in a particular context will often resemble normal features of some other protein, making it difficult to base discrimination on any single parameter.

Trends

Accurate target selection during protein QC often requires multiple steps involving repeated client surveillance, provisional recognition, and commitment reactions.

Irreversible reactions that prevent further engagement of biosynthetic machinery can effectively commit targets to degradation well before ubiquitin ligases are recruited.

Competition between maturation and QC machineries is avoided by temporarily prioritizing biosynthesis. This advantage is conferred by a combination of spatial proximity, factor abundance, and faster kinetics.

Glycan trimming in the ER provides a slow but irreversible timer that measures ER residence time and prioritizes substrates between different fates.

Kinetic proofreading by competing enzymatic reactions can dramatically sharpen discrimination of subtly different interaction affinities between clients and QC machinery to determine client fate.

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Box 1. Protein QC and Disease

Protein QC is a heavily studied field in the biomedical sciences because it is often associated with disease. Because QC occurs in all cells, the range of QC-linked diseases is especially broad, encompassing cardiovascular disease, lung disease, metabolic disorders, and some cancers [2,3]. However, the most notable QC-linked diseases have neurodegenerative phenotypes, probably because neurons are particularly long-lived and are therefore especially sensitive to the accumulation of even slight perturbations in protein homeostasis over time. Mutations involving protein QC can lead to disease in several ways, as outlined below. In addition, decline in QC with aging or adverse environmental conditions are also thought to be contributing factors in various diseases.

QC Target Gain-of-Function

Inherited mutations can occur in QC targets to cause a gain-of-function disease phenotype. In these cases, mutated proteins that should be recognized by QC and targeted for degradation escape at some low rate. As a result, they accumulate and cause pathologic consequences that are unrelated to the normal function of the protein. This mechanism is associated with many late-onset neurodegenerative disorders, such as Huntington's and related polyglutamine expansion diseases [101], and familial forms of Alzheimer's and Parkinson's disease [2]. Other examples include amyloidosis linked to cataracts, diabetes, and cancers [2].

QC Target Loss-of-Function

In other cases, mutations may leave the biochemical activity of a protein partially or completely intact, but nonetheless cause the mutant protein to be prematurely recognized and degraded by QC machinery. This leads to the loss of its normal function within cells. This mechanism is associated with degradation of important transporters and enzymes, including certain mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) protein linked to cystic fibrosis [102], and some mutations in lysosomal hydrolases that cause lysosomal storage disorders [103].

Defects in QC Machinery

Finally, disease-causing mutations may occur in QC machinery. Complete loss of many QC components, such as ubiquitin ligases and chaperones, is often embryonic lethal or causes severe phenotypes in model organisms, and presumably also in humans. By contrast aberrant expression or hypomorphic alleles of QC machinery components have been linked to numerous human diseases, including genetic disorders, neurodegeneration, immune system function, and cancers [104,105].

Second, *bona fide* QC substrates are typically rare relative to qualitatively similar normal counterparts. Detecting rare substrates is complicated because even infrequent engagement of vastly more abundant normal versions can provide substantial competition. Thus, when the ratio of abnormal to normal is relatively low, as would be ideal for cellular homeostasis, the burden on discriminatory power becomes high.

Third, all polypeptides are initially synthesized in an unfolded state and must undergo folding, modification, localization, and assembly to achieve a functional mature state. These maturation intermediates, although non-functional, must be provisionally protected from degradation. However, after a grace period has passed, they must be detected as failed maturation products by QC machinery [7]. Thus, QC pathways should be capable of measuring time to prioritize their potential clients and avoid interfering with the essential process of protein maturation.

To overcome these challenges, protein QC pathways rely on the combinatorial use of biochemical features, local context, cellular location, and time to selectively target aberrant proteins for degradation. Hence, target selection is often not achieved in a single discriminatory step (see Box 2 for potential counterexamples). Instead, a series of reactions involving repeated client surveillance, provisional recognition, and commitment steps are typically necessary. These key phases of target selection are discussed in the context of four different QC systems, illustrating the diverse strategies cells have evolved to meet the challenges of high-fidelity target selection.

Ribosome-Associated QC

Studies on the recently identified RQC pathway [8–13] have unveiled well-defined target recognition and commitment steps (Figure 1). The RQC pathway is responsible for degrading the partially synthesized proteins generated when ribosomes stall during translation elongation.

Glossary

Cotranslational targeting: SRP-mediated targeting of ribosome-nascent polypeptides that have ER targeting sequences to the ER, where the nascent polypeptide subsequently translocates into the lumen or inserts into the membrane as it is being translated.

Deubiquitinase (DUB): a protease that cleaves ubiquitin from proteins.

Dislocation: the process of extracting ER proteins from the lumen or membrane into the cytosol for proteasomal degradation.

Dislocation is usually signaled by ubiquitination and minimally requires a membrane conduit and the Cdc48/p97 AAA ATPase complex.

E3 ubiquitin ligase: enzymes that catalyze the covalent attachment of ubiquitin to a substrate that is either directly or indirectly associated with the ligase.

ER-associated degradation (ERAD): an umbrella term for protein QC pathways within the ER that target proteins for dislocation and proteasomal degradation.

Glucosidase: an enzyme that removes glucose residues from glycans.

Glycosylation: the process of adding glycans to proteins in the ER. This modification can occur co- or post-translationally.

Mannosidase: an enzyme that removes mannose residues from glycans.

Mislocalized protein: proteins that have failed to target to their correct cellular compartment. In the context of this review, this term specifically refers to proteins containing hydrophobic ER targeting sequences but which are present in the cytosol.

Post-translational targeting: delivery of proteins containing hydrophobic ER targeting signals to the ER after these proteins are completely translated from ribosomes.

Proteasome: a large protein complex found in cytoplasm and nuclei that degrades proteins. Delivery to the proteasome is typically via ubiquitin molecules on the substrate.

Ribosome-associated quality control (RQC): a protein QC pathway that degrades the partially synthesized nascent polypeptides produced by ribosomes that have stalled during translation elongation.

Box 2. Target Selection by Ubiquitin Ligases

The earliest concept of target selection during QC involved direct recognition of targets by a ubiquitin ligase, perhaps with the aid of an adaptor. Although it is now apparent that ubiquitination often acts after targets have already been selected in many pathways, direct recognition by ligases is nevertheless an important mechanism in some cases.

Direct Recognition by Ubiquitin Ligases

Several ubiquitin ligases, elucidated primarily in the yeast model system, may directly interact with substrates in a misfolding-dependent manner. Ubr1 (ubiquitin protein ligase E3 component N-recognin 1) and Ubr2 can mediate degradation of cytosolic misfolded proteins [106–109]. Similarly, the nuclear E3 ligase San1 (Sir antagonist 1) is thought to interact directly with misfolded nuclear and cytosolic proteins via its own intrinsically disordered region [110]. Although client recognition is thought to be direct in these systems, this remains to be rigorously analyzed. Membrane-embedded E3 ligases such as Hrd1 can also engage clients directly in addition to their use of adaptors [88,96]. The extent to which these direct interactions are the sole point of discrimination is not known. It is worth noting that caution is warranted in inferring discrimination solely from direct interactions measured with purified components. The absence of numerous potential competitors may exaggerate or blunt discrimination relative to a physiologic context.

Recognition via Adaptor Proteins

Adaptor proteins can further broaden the substrate range and selectivity of ubiquitin ligases. Because chaperones are ideally suited to recognizing unfolded domains, their use as a ligase adaptor has long been an attractive idea [111]. The ubiquitin ligase CHIP (C-terminal Hsp70-interacting protein) interacts with Hsp70 and Hsp90 [112]. However, its role in QC has been extensively debated, and most evidence for this view comes from overexpression studies, with loss of CHIP causing a surprisingly modest phenotype [113]. Better-defined examples include the recently discovered system for degrading selenoproteins that fail to incorporate selenocysteine at a UGA codon. Inappropriate termination at this codon exposes a degon of ~10 preceding residues that is recognized by adaptor proteins for the cullin ring ligase CRL2 [114]. Finally, the ligase Rsp5 (reverses Spt phenotype5; Nedd4 in mammals) also uses interchangeable adaptor proteins [the ART (arrestin-related trafficking adaptor) family] to recognize its plasma membrane QC targets [115]. In addition, Rsp5 can associate with the Hsp40 co-chaperone Ydj1 (yeast dnaJ) to ubiquitinate cytosolic proteins during heat shock [116].

Stalling can occur for several reasons and can initiate a response that degrades the mRNA [14–18], nascent polypeptide [8,19], and possibly even the ribosome [20].

Degradation of the partially synthesized polypeptide requires the **E3 ubiquitin ligase** Ltn1 (Listerin) [8]. Because Ltn1-mediated degradation is specific to translationally arrested polypeptides, it was initially thought that Ltn1 might specifically recognize stalled ribosomes, raising the question of how active ribosomes are avoided. Surprisingly, the mechanism for target selection turned out to not involve the ligase at all. Instead, biochemical reconstitution studies showed that splitting of stalled 80S ribosomes is a prerequisite for Ltn1 recruitment and nascent chain ubiquitination [11]. This implicated the ribosome splitting machinery in selecting which nascent polypeptides are eventually targeted for degradation.

Splitting of stalled ribosomes is mediated by the GTPase Hbs1 (Hsp70 subfamily B suppressor), its interacting partner Dom34 (duplication of multilocus region34; Pelota in mammals), and the ATPase Rli1 [RNaase L inhibitor; ABCE1 (ATP-binding cassette sub-family E member 1) in mammals] [21,22]. Hbs1 is homologous to other **translational GTPases** such as the eukaryotic elongation factor eEF1A and the eukaryotic release factor eRF3, whereas Dom34 is homologous to eukaryotic release factor eRF1. Both Dom34 and eRF1 are roughly shaped like a tRNA. Thus, the complexes that mediate elongation (aminoacyl-tRNA-eEF1A), termination (eRF1–eRF3), and splitting of stalled ribosomes (Dom34–Hbs1) share features that provide each with access to the GTPase center and A site of the ribosome [23]. It can therefore be deduced that surveillance of the state of the ribosome–mRNA complex is primarily determined by the relative abundances, affinities, and specificities of these three GTPase complexes, each of which mediates a different outcome.

GTP-bound eEF1A, eRF3, or Hbs1 each deliver their binding partner to an empty A site. This provisional interaction can dissociate unless the bound GTP is hydrolyzed. Hydrolysis permits

Sec61 translocon: a heterotrimeric ER membrane protein complex that mediates the translocation of proteins into the ER lumen and the insertion of transmembrane domains (TMDs) into the ER membrane.

Signal recognition particle (SRP): a ribonucleoprotein complex that binds to hydrophobic ER targeting sequences on ribosomes to target those ribosomes to the ER.

Signal sequence: an N-terminal hydrophobic sequence that targets proteins to the ER by engaging the targeting factor SRP as soon as it emerges from the ribosome. At the ER, the signal sequence engages the Sec61 translocon and is usually cleaved during protein translocation.

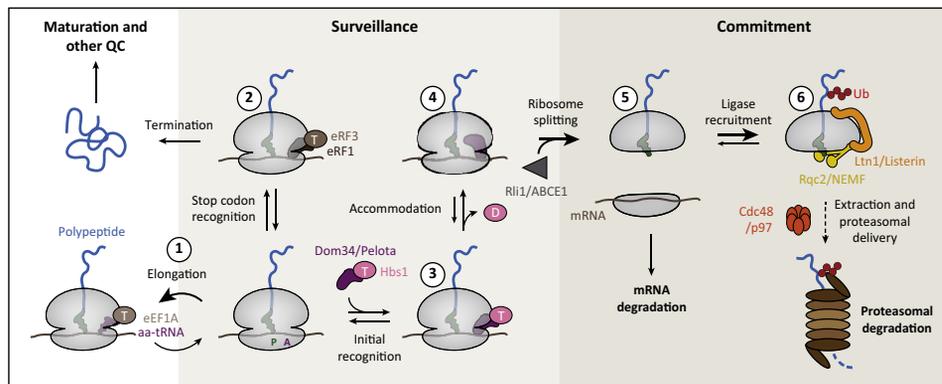
Tail-anchored (TA) proteins: membrane proteins with a single C-terminal TMD. They are post-translationally targeted to the membrane for insertion.

Translational GTPase: GTPase that interacts with ribosomes and hydrolyzes GTP to effect key reactions needed for protein translation.

Triage: refers to the process of prioritizing a protein between different fates.

Ubl domain: ubiquitin-like domain often used for protein–protein interactions, most notably with the proteasome.

UDP-glucose:glycoprotein glucosyltransferase (UGGT): a soluble enzyme within the ER lumen that specifically recognizes unfolded glycoproteins and transfers a glucose molecule onto the terminal mannose of protein glycans.



Trends in Biochemical Sciences

Figure 1. Ribosome-Associated Quality Control. Translation elongation (1) cycles between states in which the ribosomal A site is occupied most of the time, but transiently empty. Sense codons in the A site will engage the appropriate eEF1A-aminoacylated tRNA (aa-tRNA) to continue translation elongation, whereas stop codons will recruit the eukaryotic release factors eRF3–eRF1 (2) for translation termination. If these biosynthetic factors cannot properly engage, then GTP-bound (T) Hbs1–Dom34 can bind (3). GTP hydrolysis by Hbs1 allows Dom34 (Pelota in mammals) to accommodate into the A site and GDP-bound (D) Hbs1 to leave (4). This configuration recruits the ATPase Rli1 (ABCE1 in mammals) to mediate ribosome splitting into the large and small subunits. This commits clients to pathways that degrade both the mRNA and the partially-synthesized nascent chain. Long nascent chains still attached to the P site tRNA (green) remain trapped on the large ribosomal subunit after splitting (5). This complex is specifically recognized by Rqc2 (NEMF in mammals), which recruits the E3 ubiquitin ligase Ltn1 (Listerin) to ubiquitinate the nascent chain (6). Subsequently, the polyubiquitinated nascent chain is extracted from the ribosome in a reaction that requires Rqc1 (TCF25 in mammals) and the Cdc48 (p97) complex for proteasomal degradation. Abbreviations: eEF1A, eukaryotic elongation factor 1A; NEMF, nuclear export mediator factor; TCF25, transcription factor 25.

the binding partner (aminoacyl-tRNA, eRF1, or Dom34) to ‘accommodate’ stably into the A site concomitant with release of the GTPase [23]. Importantly, the GTPase activity of eEF1A or eRF3 is stimulated when the aminoacyl-tRNA or eRF1, respectively, makes key interactions with the mRNA codon in the A site. By contrast, the GTPase activity of Hbs1, although reliant on Dom34 and the ribosome, appears impervious to the presence or identity of the mRNA within the A site [21,22,24,25].

Accommodation is a decisive event. Accommodated aminoacyl-tRNA or eRF1 typically ensures peptide bond formation or nascent chain release, respectively [23], and both accommodated eRF1 and Dom34 recruit Rli1 to drive subunit separation [22,26–29]. Thus, successful target selection in the RQC pathway appears to be dictated by the accommodation of Dom34 preferentially in stalled but not elongating or transiently paused translation complexes. Accommodated Dom34 would preclude other GTPase complexes from binding to the A site and favor Rli1-dependent ribosome splitting, which is an irreversible reaction that initiates downstream steps of the RQC pathway.

Although rigorous demonstration awaits future analyses, it seems plausible that selectivity for stalled ribosomes will reside in the intrinsic timer imposed by Hbs1 GTPase activity relative to competition by other complexes. In this model, the GTP–Hbs1–Dom34 complex can sample ribosomes whose A site and GTPase center are not already occupied. Hence, actively translating ribosomes may permit very few sampling opportunities owing to abundant elongation factors outcompeting Hbs1–Dom34. However, when codon recognition is slow (or not possible; for example, if an mRNA is truncated), Hbs1–Dom34 can bind. The time before GTP hydrolysis and Dom34 accommodation permits a window of competition if a more suitable complex becomes available.

In addition to eEF1A and eRF3 complexes, other possible competitors, such as deacylated tRNAs that accumulate under conditions of amino acid starvation [30], may allow the cell to differentiate between physiologic pauses that should not result in ribosome splitting from pathologic stalls. The discovery that GTP binding protein 2 (GTPBP2), a close homolog of Hbs1, also interacts with Pelota and is necessary to resolve ribosome stalls due to defects in a neuron-specific tRNA [31], suggests that GTPBP2 and Hbs1 functions are non-redundant. This implies that additional layers of specificity may be encoded in this ribosome recognition step. The features that determine these differences await future studies.

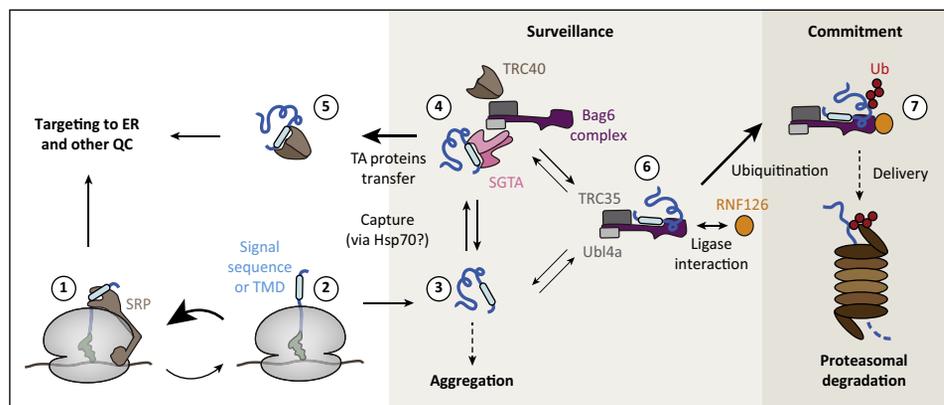
Once the ribosome is split, the nascent chain housed within the large ribosomal subunit is effectively committed to degradation. Translation is no longer an option, and each subsequent reaction is efficiently driven by the preceding step. Removal of the small subunit exposes a unique interface formed by the juxtaposition of a P site tRNA with the intersubunit surface of the 60S. The exposed tRNA and 60S surface are specifically and simultaneously recognized by NEMF (nuclear export mediator factor, Rqc2 in yeast) [13,32,33], but neither element alone is sufficient for stable NEMF interaction [13]. Ltn1 is then recruited by simultaneously interacting with NEMF and multiple positions on the ribosome [13]. Subsequent ubiquitination of the polypeptide mediated by the RING domain of Ltn1, which is positioned adjacent to the ribosomal exit tunnel, is highly processive. The downstream steps leading to degradation by the **proteasome** have not been mechanistically resolved, but require recruitment of the Cdc48 complex via the polyubiquitinated client and the ribosome-associated factor Rqc1 [9,10,12].

Thus, surveillance in the RQC pathway exploits factors that mimic biosynthetic counterparts. These factors do not seem to recognize a specific 'defect' but instead act by default on translation complexes that are not successfully engaged by translation factors in a timely manner. Commitment is determined by a biochemical reaction, ribosome splitting, which simultaneously precludes further translation and generates a novel cue for downstream steps in the pathway. Remarkably, in this system, the polypeptide to be degraded is not recognized directly but inferred from surrogate cues, and the ubiquitin ligase apparently has no decision-making capacity in selecting QC targets. Instead, polyubiquitination serves as a molecular handle for extraction and proteasome targeting.

QC of Mislocalized Proteins

QC pathways must integrate into biosynthetic pathways such that potential clients can be prioritized between maturation and degradation. Ideally, degradation should be favored only after maturation has failed, but nevertheless occur efficiently. The QC of mislocalized proteins in metazoans provides an example of multi-step prioritization whose mechanisms are now beginning to emerge (Figure 2). This pathway monitors protein targeting to the endoplasmic reticulum (ER) and **triages** for degradation any polypeptides that fail [34].

Protein targeting to the ER is mediated by transmembrane domains (TMDs) or cleavable N-terminal **signal sequences** [35–37]. These hydrophobic elements are recognized by targeting factors that simultaneously shield them from the cytosol and deliver them to receptors at the ER membrane. If the targeting sequence emerges from the ribosome during translation, it is recognized by a ribosome-bound **signal recognition particle** (SRP) for **cotranslational targeting** [36,38]. If the sole TMD is close to the C terminus, it cannot engage SRP cotranslationally and instead uses a **post-translational targeting** factor termed TRC40 (TMD recognition complex 40 kDa subunit) [37,39]. Targeting can sometimes fail, generating a mislocalized protein that should be degraded by the proteasome. These mislocalized proteins are recognized via their hydrophobic targeting elements by Bag6 (BCL2-associated athanogene 6), which recruits the E3 ligase RNF126 (ring finger protein 126) for client ubiquitination [34,40].



Trends in Biochemical Sciences

Figure 2. Quality Control of Mislocalized ER Proteins. Ribosomes translating proteins with hydrophobic endoplasmic reticulum (ER) targeting sequences are usually recognized by the signal recognition particle (SRP) (1). SRP binds to both the targeting sequence and the ribosome to deliver clients cotranslationally to the ER. ER proteins that fail to or cannot engage SRP (2) are released into the cytosol (3). If left unattended, these hydrophobic proteins will tend to aggregate. Specialized chaperones, the most upstream of which appears to be SGTA (Sgt2 in yeast), capture these proteins to shield their hydrophobic elements (4). SGTA interacts with the heterotrimeric Bag6 complex, which bridges SGTA with the post-translational targeting factor TRC40 (5). **Tail-anchored (TA) proteins** that have their sole transmembrane domain (TMD) at their C-terminus are transferred to TRC40 (5) for post-translational delivery to the ER. Otherwise, mislocalized proteins are captured by the Bag6 protein (6), which recruits the ubiquitin ligase RNF126 via an N-terminal Ubl domain. RNF126 mediates client ubiquitination (7), which eventually commits them to proteasomal degradation. Abbreviations: SGTA, small glutamine-rich tetratricopeptide repeat-containing protein alpha; TRC40, TMD recognition complex 40 kDa subunit; Ubl, ubiquitin-like.

The selectivity of Bag6 for relatively lengthy hydrophobic domains mirrors the specificity of SRP and TRC40 [34,41]. This explains how Bag6 discriminates proteins that should have been captured by one of these targeting factors from nascent cytosolic proteins, but raises the question of how Bag6 can use the same elements needed for targeting to mediate degradation without competing with targeting factors. Although not fully resolved yet, the solution appears to depend on a set of spatially constrained hierarchical interactions.

At the top of this hierarchy is SRP, which captures substrates on the ribosome and therefore has the opportunity of first refusal [36,38,42]. Although Bag6 also associates with ribosomes, it cannot interact with clients cotranslationally [34,41,43]. Upon release from the ribosome, TMD-containing proteins appear to favor engagement of a chaperone-like protein termed SGTA (small glutamine-rich tetratricopeptide repeat-containing protein alpha, Sgt2 in yeast) [44]. The basis of this preference is unclear, but it is noteworthy that Sgt2 has conserved tetratricopeptide repeat (TPR) motifs that can associate with the abundant chaperone Hsp70 (heat shock protein of 70 kDa) [44]. Given that nascent chains can bind Hsp70 cotranslationally [45], they may be able to sample SGTA/Sgt2 before other TMD-binding factors. In yeast, Sgt2 clients can be transferred to the TRC40 homolog Get3 (guided entry of tail-anchored proteins 3) via the Get4–Get5 bridging complex, with Get5 recruiting Sgt2 and Get4 recruiting Get3 [44,46,47]. A very similar reaction occurs in metazoans, with one key exception: the bridging complex includes Bag6. The mammalian Get5 homolog (Ubl4A, ubiquitin-like 4A) does not associate directly with its Get4 homolog (TRC35), but is instead bridged by the C-terminal ~100 residues of Bag6 [41,48,49]. Thus, client transfer from SGTA to TRC40 necessarily occurs in proximity to Bag6.

Bag6 is a long ~1200 residue protein. Whereas its C terminus mediates substrate transfer to TRC40 by forming a complex with Ubl4A and TRC35 [48,49], its N terminus contains the

ubiquitin-like domain (Ubl domain) that recruits RNF126 [34,40], and substrate binding occurs via a poorly-defined middle region [50]. Thus, the architecture of the SGTA–Bag6–TRC40 complex suggests a plausible model for substrate triage between targeting and degradation. If the client contains a single C-terminal TMD, it would favor transfer to TRC40 [39,41]. This is consistent with the known client preference of TRC40 [39,51] and the very rapid kinetics of this reaction deduced from the yeast system [47,52,53]. Loading onto TRC40 is ostensibly a commitment to targeting because substrate binding may facilitate dissociation from TRC35 (as deduced from the yeast counterparts) [53]. Thus, the availability of TRC40 and a suitable substrate would prioritize targeting as the favored outcome.

If TRC40 is unavailable or the substrate is not optimal (i.e., a protein with an internal or multiple hydrophobic domains), Bag6 binding is favored [34,41]. One might postulate that this is the default, albeit slower, outcome that can only be escaped by a more rapid transfer to TRC40. Bag6 interaction may provisionally commit substrates to degradation, consistent with its ‘holdase’ activity [54]. Bag6 may serve an analogous function of holding hydrophobic clients at a step downstream of commitment during **ER-associated degradation** (ERAD) [55–57]. The N-terminal Ubl domain of Bag6 recruits RNF126 to ubiquitinate the bound client [40], and the combination of client ubiquitin and the Ubl domain may facilitate proteasomal targeting [58].

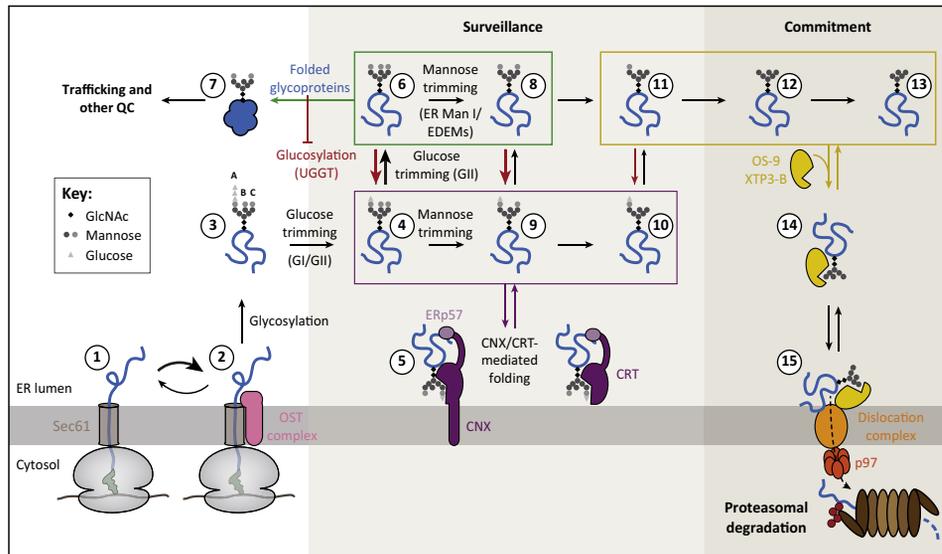
Surveillance for mislocalized proteins therefore appears to be mediated by a relatively promiscuous and moderately abundant factor (Bag6) that recognizes elements that should have been engaged by targeting factors (SRP or TRC40). Degradation is probably the default outcome of engaging Bag6, a fate that is avoided by engaging SRP or TRC40 first. Priority is apparently encoded by the spatial advantage imparted by the position of SRP on the ribosome and a kinetic advantage for client transfer from SGTA to TRC40. Commitment is therefore a late step in this pathway, potentially occurring only when the client is ubiquitinated or targeted to the proteasome.

Glycoprotein QC in the ER

One of the largest challenges of protein QC is the need to distinguish QC targets from folding intermediates, which should be protected from degradation and given opportunities to mature. Because yet-to-be-folded polypeptides are biochemically similar to those that have tried and failed, commitment to degrade the latter must simultaneously assay both polypeptide structure and elapsed time. The QC of nascent glycoproteins in the ER lumen provides the best-studied example of how time can be encoded to facilitate client selection relative to biosynthetic intermediates (Figure 3) [59,60].

A central player in this QC pathway is the asparagine-linked glycan that is covalently transferred by the oligosaccharyltransferase (OST) complex onto substrates exposing a **glycosylation** consensus sequence in the ER lumen [61]. The 14-sugar core glycan consists of a base of two *N*-acetylglucosamine (GlcNAc) residues, nine mannose residues that form three branches (termed A, B, and C), and three glucose residues appended to the A branch. Once a protein is glycosylated, the two outermost glucose residues are rapidly trimmed by **glucosidases** I (GI) and II (GII) [59,60], respectively, to generate a monoglucosylated core glycan.

The monoglucosylated glycan, possibly together with features of the polypeptide chain, is specifically recognized by the lectin-chaperones calnexin (CNX) and/or calreticulin (CRT) [62]. CNX/CRT can also associate with the oxidoreductase ERp57 (endoplasmic reticulum resident protein 57, also known as PDIA3) and the peptidyl-prolyl isomerase cyclophilin B, thereby recruiting them to the nascent polypeptide [60,62]. Dynamic interactions with such chaperone complexes presumably minimize off-pathway interactions while facilitating acquisition of native



Trends in Biochemical Sciences

Figure 3. Glycoprotein Quality Control. Most glycoproteins are cotranslationally targeted to the **Sec61** translocon for insertion or translocation into the ER (1). Proteins with consensus glycosylation sites are glycosylated by the translocon-associated oligosaccharyltransferase (OST) complex (2). The asparagine-linked 14-sugar core glycan (3) is immediately trimmed by glucosidases I (G1) and II (GII) to generate a monoglucosylated substrate (4) that can access calnexin (CNX)- and/or calreticulin (CRT)-mediated folding cycles (5). GII can also further remove the last glucose from substrate glycans to generate clients (6) that can be trafficked out of the ER if they are properly folded (7). Otherwise, unfolded glycoproteins are recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT), which can add back a glucose onto the glycan (4) such that clients can re-engage CNX/CRT (5). Resident mannosidases, such as ER mannosidase I (ER Man I) and the ER degradation-enhancing α -mannosidase-like proteins (EDEMs), irreversibly trim mannose off from unglucosylated (4,9) or monoglucosylated (6,8,11,12) clients. These trimmed clients remain in the glucosylation cycle mediated by GII and UGGT (8–11) for a short period of time, providing them with additional opportunities to access the CNX/CRT cycle (5) if they are monoglucosylated (9,10), or to leave the ER if they are unglucosylated (8) and properly folded. However, mannose trimming beyond a particular point (12,13) renders substrates incapable of re-engaging GII or UGGT. These clients (11–13) have no further chances to leave the ER and are instead recognized by the OS-9 (Yos9 in yeast) or XTP3-B lectins (14) that deliver clients to the dislocation machinery (15) for degradation.

structure. When the glycan is not engaged by CNX/CRT, the remaining glucose residue is susceptible to removal by GII [59,60,62], which initiates the first key decision point in this pathway. If the polypeptide has folded, it can potentially traffic out of the ER via bulk flow or export receptors, some of which are also lectins [63]. Many substrates fold rapidly and leave the ER on the first instance of glucose removal [64].

If the nascent glycoprotein has not folded yet, it should ideally re-engage chaperones. For CNX/CRT, this requires re-glucosylation by **UDP-glucose:glycoprotein glucosyltransferase** (UGGT) [59,60,65]. UGGT is a key component of glycoprotein surveillance, deciding whether clients re-engage CNX/CRT based on their folding status. The mechanism of this determination is not fully resolved, but seems to involve two criteria. First, client recognition by UGGT seems to involve exposed hydrophobic patches indicative of unfolded or unassembled proteins [66–69]. Second, the glycan to be re-glucosylated appears to bind to UGGT in part via its GlcNAc residues at the glycan base [70]. The close proximity of GlcNAc to the attached protein may necessitate some local polypeptide flexibility, which is another indicator of unfoldedness. Thus, slow-folding glycoproteins undergo cycles of glucosylation (by UGGT) and deglycosylation (by GII) to retain them in the ER (via CNX/CRT interactions) for multiple opportunities to fold [65,71].

However, proteins that fail to fold need to be removed from futile folding cycles and triaged for degradation. This essential timing decision appears to be mediated by ER-resident **mannosidases** such as ER mannosidase I and the ER degradation-enhancing α -mannosidase-like (EDEM) proteins [72–76]. Mannose trimming by these enzymes is a slow but irreversible process that effectively measures ER residence time. Both mammalian EDEM and the yeast homolog Htm1 (homologous to mannosidase 1) are found in complexes with oxido-reductase-chaperones [77,78], suggesting that client engagement and demannosylation might be influenced by folding status. Sequentially removing mannose residues from the core glycan makes substrates successively poorer clients for UGGT and GII, eventually rendering them incapable of re-engaging the glucosylation/de-glucosylation cycle once the terminal mannose on the A branch is removed [79].

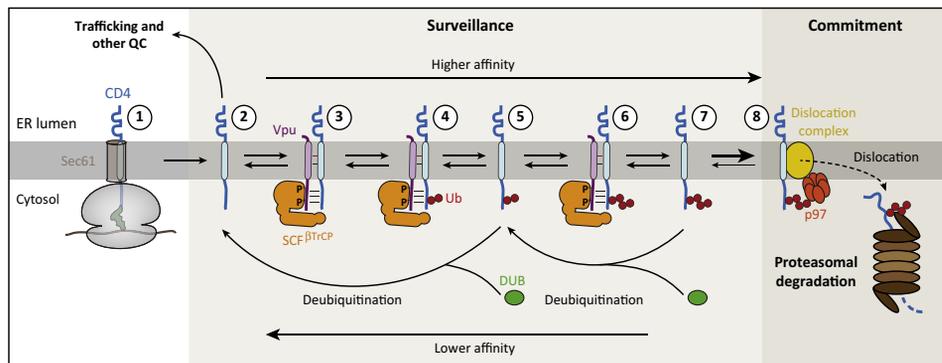
Substrates with an expired ‘mannose timer’ are effectively committed to degradation because they are prevented from further attempts at maturation. Instead, an exposed α 1,6 mannose linkage on the trimmed glycan is recognized by the OS9 (osteosarcoma amplified 9; Yos9 in yeast)/XTP3-B [XTP3-transactivated gene B protein or endoplasmic reticulum lectin 1 (ERLEC1)] lectins [80–85], which mediate their delivery to **dislocation** machinery to initiate the degradation process [85–87]. In addition to OS-9/XTP3-B-mediated targeting, the dislocation complex may also query the folding status of the polypeptide directly [88]. This dual requirement would sharpen client discrimination by precluding promiscuous degradation of either folded proteins or maturing proteins whose mannose timer has not expired.

Thus, during QC of glycoproteins, the rates of glycan trimming mark time and collude with structure-dependent interactions to drive commitment toward successful maturation or degradation. Cycles of glucose addition and removal combined with glycan-mediated folding give challenging substrates multiple opportunities to mature, while slow but irreversible mannose trimming limits the number of folding opportunities before substrates are deemed defective [60,76]. Importantly, this entire process is mediated by ongoing competition between the various lectins and glycoprotein-modifying enzymes because the binding of one factor to a substrate glycan is presumably mutually exclusive with that of another. This suggests that the length of the ‘mannose timer’ is not absolute for all proteins. Substrates that have higher affinity for UGGT and/or CNX/CRT would minimize their interactions with GII and mannosidases, giving them a longer time-window for maturation.

Although less well-studied, a second ‘timer’ might be imparted by the slow mannosylation of serine and threonine residues by ER-resident mannosyl transferases. In this instance, modification is again irreversible and has the consequence of precluding further folding attempts, thereby indirectly committing the protein for degradation [89]. This idea has only been demonstrated with an artificial substrate, and the extent to which this timer is used generally remains to be investigated. Finally, sub-compartmentalization of the various factors involved in glycoprotein QC [72,76] might further sharpen the target selection or commitment steps, but this also remains poorly studied.

Kinetic Proofreading during QC

Discriminating subtly different conformational states for a wide range of proteins poses a considerable challenge for protein QC. In some cases, engagement with a ubiquitin ligase might be sufficient for QC target selection (Box 2). However, many general QC ligases must make fine distinctions based on poorly defined criteria. How small differences in interaction are converted to decisive outcomes is a common but poorly understood problem in QC. Recently, detailed analysis of a simplified model system for client recognition and ubiquitination has demonstrated how kinetic proofreading can sharpen the discernment of such subtle differences (Figure 4).



Trends in Biochemical Sciences

Figure 4. Kinetic Proofreading Sharpens Target Discrimination. CD4 is cotranslationally inserted into the ER membrane via the Sec61 translocon (1). CD4 is normally trafficked to the cell surface (2). However, the HIV protein Vpu can associate with CD4 via interactions within their transmembrane and cytosolic domains (3). Vpu recruits the Skp, Cullin, F-box containing ubiquitin ligase complex bound to the F-box protein β TrCP ($SCF^{\beta TrCP}$) via its phosphorylated cytosolic tail (3) to mediate ubiquitination of CD4 (4). Vpu and CD4 can undergo cycles of dissociation (5,7) and reassociation (3,6). Dissociation favors ubiquitin removal by deubiquitinases (DUBs), whereas reassociation favors ubiquitin addition. Thus, relatively short interactions between CD4 and Vpu will drive the reaction back towards the ground state (2), with only brief residence time of a polyubiquitinated state. By contrast, longer interaction times drive more-processive ubiquitination (7) and increased dwell time of the polyubiquitin chain. Polyubiquitin can engage downstream components, such as the p97 complex, that mediate dislocation of CD4, thereby committing it for proteasomal degradation (8).

The model system is based on the ability of the small HIV-encoded membrane protein Vpu (virus protein U) to mediate degradation of newly synthesized CD4 (cluster of differentiation 4) from the ER [90]. Vpu interacts with CD4 at the ER and mediates CD4 ubiquitination by recruiting $SCF^{\beta TrCP}$ (Skp, Cullin, F-box containing ubiquitin ligase complex bound to the F-box protein β TrCP) via its phosphorylated cytosolic tail [91]. Point mutations that only slightly weaken the interaction between Vpu and CD4 dramatically impair Vpu-mediated CD4 ubiquitination and degradation in cultured cells. However, when Vpu-mediated CD4 ubiquitination was reconstituted with purified components in liposomes, the same mutations had almost no discernible effect [92], suggesting that additional factors are required for effective client discrimination.

This missing component turned out to be the activity of **deubiquitinases** (DUBs) that constantly counteract ubiquitination. Adding a promiscuous DUB to the purified system was sufficient to enhance the difference in polyubiquitination between wild-type and mutant interactions by 10-fold [92]. Conversely, inhibiting DUBs *in vitro* or in cells blunted differences in poly-ubiquitination between CD4 clients with different affinities for the Vpu– $SCF^{\beta TrCP}$ ubiquitin ligase complex. These observations together with kinetic modeling led to a working framework for how competing cycles of ubiquitination and deubiquitination are able to convert modest differences in ligase–client interaction into large differences in polyubiquitination (and by inference, large differences in degradation).

In this model, the length of time a client associates with the ligase determines how many ubiquitins are attached in any given encounter [93]. Upon dissociation, there is a competition between ubiquitin removal by DUBs and ligase reassociation. If reassociation with the ligase occurs before all ubiquitins are removed, the overall number of ubiquitins would increase. Otherwise, DUBs will return the client to its ground state (i.e., no ubiquitins) before the next ligase encounter. Thus, the relative length of time the client spends in the ground versus the ubiquitinated state is highly sensitive to DUB activity relative to ligase interaction kinetics.

In the context of QC at the ER, time spent in the two states is presumably linked to opportunities for commitment toward ER exit versus degradation. Commitment to ER exit may be mediated by

engagement of coat proteins or export receptors [63] whose interactions favor the ground state due to steric occlusion by ubiquitin. By contrast, commitment to degradation might be imparted by a polyubiquitin-binding factor, such as the Cdc48 complex, that initiates dislocation into the cytosol [94]. Thus, slight variations in the k_{on} and k_{off} parameters of the ligase–client interaction can significantly alter the residence times of the poly-ubiquitin degradation signal to impact client fate. Such an idea has been postulated to determine the timing and order of degradation of key cell cycle regulators [95].

Extrapolating these ideas to QC, one might postulate that QC ligases, such as Hrd1 (HMG-CoA reductase degradation) in the ER, have evolved to be highly promiscuous. In this view, folded proteins would be close to the ‘tipping point’ of this ubiquitination–deubiquitination balance. Hence, even subtle deviations from normal would result in degradation. This would provide the ligase with the ability to detect a wide range of deviations by simply detecting differences from what is evolutionarily-defined as ‘normal’ for that particular protein. Indeed, various QC ligase interactions with degraded versus non-degraded clients are nearly indistinguishable by relatively qualitative assays such as co-immunoprecipitation [96,97]. Furthermore, knockdown of a single ER-resident DUB was sufficient to impact degradation of a misfolded protein [98].

The use of kinetic partitioning can potentially apply to many QC systems with chaperone-associated ligases or other ligases that interact transiently with clients. An attractive feature of this strategy is that the QC ‘threshold’ for what is acceptable can be modulated in specific cell types or situations by varying DUB activity. For example, reduced DUB activity during oxidative stress might lead to more promiscuous degradation [99,100]. This may be advantageous because protein maturation and localization are more likely to fail under these stress conditions, thereby warranting more aggressive QC.

Concluding Remarks

The diversity of strategies presented by these four examples highlights several unmet goals (see Outstanding Questions) and underscores three key points. First, there is no single paradigm for target selection during protein QC. The proteome is simply too diverse and the definitions of normal and abnormal are context-dependent. Hence, generic parameters such as exposed hydrophobicity typically play contributing, but not uniquely decisive, roles in defining QC targets. Second, ubiquitin ligases often play ancillary or, in some cases, no role in target discrimination. Thus, identifying the E3 ligase for a QC pathway, historically a central goal, is only part of the solution. Some pathways, such as RQC and glycoprotein QC, employ ubiquitination at a very late step after commitment has been imparted by upstream biochemical reaction(s). Third, most QC pathways cannot be properly understood in the absence of concurrently acting biosynthetic pathways. The two processes must necessarily be coordinated, often share factors, and can have a competitive relationship. Thus, understanding the molecular basis of QC will require not only reductionist studies but also the placement of these individual reactions into a physiologic context.

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Outstanding Questions

What is the structural basis of misfolded domain recognition by QC machinery? UGGT and other QC factors preferentially recognize misfolded proteins via degenerate polypeptide features such as exposed hydrophobicity. The structural basis for this type of molecular recognition is poorly defined.

What are the molecular mechanisms that triage proteins between biosynthesis and degradation? Triage reactions are typically dynamic, warranting kinetic studies and modeling in reconstituted systems to understand the mechanism of partitioning. Such analyses require a full accounting of the factors involved in a QC pathway, an important but unachieved goal in most cases.

How is time encoded in QC pathways? QC of glycoproteins utilizes irreversible mannose trimming to keep track of how long clients have spent trying to mature in the ER, which allows selective targeting for degradation of those that have spent too long. The mechanisms by which other QC pathways measure client age to distinguish biosynthetic intermediates from proteins that have failed to mature are unknown.

Is kinetic proofreading a widely utilized mechanism of QC pathways? Competition between ubiquitination and deubiquitination can significantly enhance discrimination of subtle differences in interaction affinities to change the fate of QC clients. Because many ubiquitin ligases have been found associated with DUBs, it is attractive to postulate their role in target selection, but this remains to be investigated.

What other QC target recognition strategies are utilized in metazoans? It is clear that metazoans have diversified QC to meet the demands of a complex proteome, widely varied cell types with different tolerances for misfolding, infrequent cell division, and longer life-spans. Elucidating yet-unidentified QC pathways in mammals and the mechanisms by which they are tuned under different conditions is crucial to understanding how protein homeostasis is maintained in health and disease.

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