UBQLN4 recognizes mislocalized transmembrane domain proteins and targets these to proteasomal degradation

Rigel Suzuki & Hiroyuki Kawahara*

Abstract

The majority of transmembrane proteins are integrated into the endoplasmic reticulum (ER) by virtue of a signal sequence-mediated co-translational process. However, a substantial portion of transmembrane proteins fails to reach the ER, leading to mislocalized cytosolic polypeptides. Their appropriate recognition and removal are of the utmost importance to avoid proteotoxic stress. Here, we identified UBQLN4 as a BAG6-binding factor that eliminates newly synthesized defective polypeptides. Using a truncated transmembrane domain protein whose degradation occurs during a pre-ER incorporation process as a model, we show that UBQLN4 recognizes misassembled proteins in the cytoplasm and targets these to the proteasome. We suggest that the exposed transmembrane segment of the defective polypeptides is essential for the UBQLN4-mediated substrate discrimination. Importantly, UBQLN4 recognizes not only the defective model substrate but also a pool of endogenous defective proteins that were induced by the depletion of the SRP54 subunit of the signal recognition particle. This study identifies a novel quality control mechanism for newly synthesized and defective transmembrane domain polypeptides that fail to reach their correct destination at the ER membrane.

Keywords BAG6; proteasome; signal sequence; SRP; ubiquitin

Subject Categories Membrane & Intracellular Transport; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

Recent progress in genome analysis revealed that more than one-fifth of proteins encoded in the mammalian genome are putative transmembrane (TM) proteins [1–4]. Since TM proteins contain an almost 20-residue long bulky hydrophobic stretch called the transmembrane domain (TMD), their biogenesis in the aqueous cytosol requires specialized machinery dedicated to TM protein assembly

[5]. Indeed, many TM proteins possess a characteristic signal sequence (SS) at the N-terminus of the nascent polypeptide for endoplasmic reticulum (ER) targeting [6–10]. The timely and accurate capture of the SS in the ribosome–nascent chain complex is essential for the proper initiation of membrane protein assembly in the ER [10]. At the initial stage of translation on cytosolic ribosomes, the SS of nascent chain polypeptides is recognized by a signal recognition particle (SRP), a novel class of GTPase whose activity is regulated by nucleotide-dependent dimerization cycles [11–15]. Subsequently, the SRP-ribosome complex is recruited to the surface of the rough ER, where newly synthesized polypeptides are assembled in the ER membrane through SRP receptor and translocon complexes [6,9,10,16].

The fidelity of SS recognition by the SRP, however, is challenged by the kinetic difficulties of targeting nascent chain polypeptides elongating from the ribosome [10,17,18]. Indeed, the SRP must identify the targeted substrates within several seconds in the cytosol; a nascent polypeptide reportedly loses its competence to be targeted by the SRP when it exceeds a critical length of 140 residues [10,19]. Furthermore, the concentration of the SRP is estimated to be more than 100-fold lower than that of the translating ribosomes [10]. Thus, difficulties of SRP recognition within a limited time window in the crowded cytosol might result in the probable production of defective SRP substrates with an orphan TMD [20,21]. Similar consequences might occur when SRP subunits are compromised or when the ratio of the SRP to its client substrates is overwhelmed [18,22].

In accordance with these studies, it was reported that some classes of TM proteins fail frequently to access the ER membrane [21,23]. Indeed, a surprisingly low efficiency of ER targeting (as low as 60% success for some newly synthesized ER proteins) was reported, resulting in the production of defective (and mislocalized) proteins [21,24,25]. Their appropriate recognition and removal before they reach the lumen of the ER are of real significance for the maintenance of cellular homeostasis [26,27]. Defects in SS machinery can lead to various human diseases such as prion disease, amyloid precursor protein-derived neurodegeneration, and autosomal dominant late-onset diabetes [28–31]. Nevertheless, the molecular mechanism of the elimination pathway that targets

Laboratory of Cell Biology and Biochemistry, Department of Biological Sciences, Tokyo Metropolitan University, Tokyo, Japan

*Corresponding author. Tel: +81 42 677 1111 (Ext.4367); E-mail: hkawa@tmu.ac.jp

newly synthesized and mistargeted TMD protein is not well understood.

A hydrophobic TMD that is normally buried in the lipid bilayer tends to be exposed in the aqueous cytoplasm by mistargeting of TM proteins during the course of their biogenesis. BAG6, a major histocompatibility complex (MHC)-encoded gene product, was identified as an essential factor in the metabolism of newly synthesized defective and/or mistargeted TM proteins [25,32–36]. The BAG6 complex recognizes the exposed hydrophobicity of TM proteins just after their ribosomal release and leads to the ER insertion of these newly synthesized polypeptides in the case of tail-anchored (TA) proteins [37–42], while it enhances the targeted degradation of the remainder (including mislocalized proteins) by the ubiquitin/proteasome-mediated degradation pathway [25,32,33]. Thus, the BAG6 complex provides an essential quality control system for TMD substrates and newly synthesized defective polypeptides with exposed hydrophobic stretches [43–47]. However, how BAG6 and its associated proteins determine the fate of client membrane proteins has not been elucidated adequately.

In this study, we explored the potential function of BAG6-associated proteins and thus identified UBQLN4 as an essential factor for the elimination of newly synthesized polyubiquitinated proteins. Using a model TMD protein whose degradation occurs at a pre-ER incorporation process, we show that UBQLN4 specifically associates with misassembled TM protein in the cytoplasm for targeted degradation via the ubiquitin pathway. Importantly, UBQLN4 recognized not only this model defective substrate but also a pool of endogenous defective proteins that were induced by compromising the SRP. We suggest that UBQLN4 acts as a substrate-discriminating ubiquitin receptor for newly synthesized and defective TM polypeptides before their ER entry.

Results

Newly synthesized polypeptides are major clients of UBQLN4

The BAG6 complex was identified recently as a receptor for TA and defective TM proteins [25,32,34,37,38,42–44,48]. During the course of our BAG6 study, we identified UBQLN4/UBIN, the main subject of this paper, as a novel binding protein of BAG6 (Appendix Fig S1). As shown in Fig EV1A, UBQLN4 co-precipitates with endogenous BAG6 protein from the extract of cells that were treated with the proteasome inhibitor MG-132, while the UBQLN4-related protein UBQLN1/PLIC1 did not. UBQLN family proteins belong to a typical class of ubiquitin-like (UBL) and ubiquitin-associated (UBA) domain proteins that can interact with proteasomes, chaperones, and polyubiquitinated species [49–57]. In accordance with the nature of UBA domain proteins [58–64], we confirmed that a significant amount of polyubiquitinated proteins co-immunoprecipitated with UBQLN4 (Fig 1A). Since co-precipitated polyubiquitinated species are induced by treatment with MG-132, the cellular function of UBQLN4 could be linked to protein degradation.

Since BAG6 reportedly associates with newly synthesized polyubiquitinated proteins that are sensitive to the protein synthesis inhibitor cycloheximide (CHX) [32], we examined whether polyubiquitinated species associated with UBQLN4 are also sensitive to CHX [65,66]. We found that treatment with CHX largely abolished

the polyubiquitin conjugates associated with UBQLN4 in MG-132 treated cells (Fig 1B). To verify further whether the UBQLN4-associated polyubiquitinated species are newly synthesized polypeptides, we performed CHX chase experiments with the time course shown in Fig 1C. The results showed that the amounts of polyubiquitinated proteins on UBQLN4 decreased successively as CHX treatment was extended (Figs 1D and EV1B). These observations suggest that a significant part of the polyubiquitinated proteins associated with UBQLN4 consists of neo-synthesized polypeptides that are targeted for proteasomal degradation just after their ribosomal synthesis.

UBQLN4 associates with cytoplasmic aggregates composed of defective polypeptides

Puromycin-induced defective nascent polypeptides reportedly tend to form ubiquitin-positive cytoplasmic aggregates called aggresome-like induced structures (ALIS) [32,67]. To verify whether UBQLN4 indeed associates with newly synthesized defective polypeptides, we performed co-immunostaining with UBQLN4 and polyubiquitin, a marker for ALIS. We found that UBQLN4 co-localized with puromycin-induced ALIS (Fig 2A, arrowhead), supporting the hypothesis that UBQLN4 possesses an intrinsic affinity for newly synthesized defective polypeptides.

To estimate the impact of UBQLN4 on the formation of ALIS, we compared the number of ALIS in the presence or absence of *UBQLN4* small interfering RNA (siRNA). We found that *UBQLN4* knockdown (using two independent double-stranded siRNAs) stimulated the formation of polyubiquitin-positive ALIS with puromycin treatment (Fig 2B and C). Additionally, *UBQLN4* knockdown accelerated puromycin-induced cell death (Fig 2D), indicating that UBQLN4 has a protective role against defective polypeptide-induced cell toxicity. To examine the possible off-target effects of *UBQLN4* knockdown on other UBQLN family proteins, we examined the effects of our double-stranded siRNAs for *UBQLN4* on the levels of UBQLN1 and UBQLN2 proteins (UBQLN3 is not expressed in HeLa cells). We provided experimental evidence that *UBQLN4* siRNAs did not interfere with the expression of endogenous UBQLN1 nor UBQLN2 proteins (Fig EV1C and D), while endogenous UBQLN4 was clearly knocked down (Fig EV1C and D, arrowheads). Thus, it is obvious that *UBQLN4* knockdown scarcely affects the expression of other UBQLN family proteins. Collectively, these results indicate that UBQLN4 is an essential element for the metabolism of aggregation-prone neo-synthesized polypeptides.

Establishing a model defective TMD polypeptide that is degraded immediately after its synthesis

Recently, the interesting concept of mislocalized proteins (MLPs) was suggested [21,25,68]. An MLP is a kind of newly synthesized defective protein that fails to be assembled correctly in the ER membrane, thus being targeted to the BAG6-mediated protein degradation pathway in the cytosol [25,68]. To explore whether such MLPs can be utilized as potential clients of UBQLN4, we examined a model for a misassembled TM protein.

The IL-2 receptor α -subunit (IL-2R α) is a single-pass TM protein that contains a typical SS for ER insertion at its N-terminus and a TMD at its C-terminus (Fig 3A). Eisenlohr and colleagues reported

Figure 2. UBQLN4 suppresses puromycin-induced ubiquitin-positive aggregates.

- A** UBQLN4 co-localizes with cytoplasmic aggregates after puromycin treatment (indicated by arrowheads). HeLa cells expressing Flag-tagged UBQLN4 were treated with 5 μ g/ml puromycin for 2 h and then followed by 1% Triton X-100 extraction and 4% paraformaldehyde fixation. Fixed cells were stained with anti-polyubiquitin FK2 (shown in red to detect ubiquitin-positive cytoplasmic aggregates, ALIS) and anti-Flag antibodies (shown in green).
- B, C** Puromycin-treated cells with UBQLN4 siRNA contain increased number of ALIS. At 72 h after transfection of the two distinct siRNA duplexes for UBQLN4 (UBQLN4 siRNA#1 and #2) or control siRNA (5 nM each), the cells were treated with 5 μ g/ml puromycin for 2 h and then subjected to immunostaining with an anti-polyubiquitin FK2 antibody (shown in green, B) as in (A). The number of ALIS was determined. The quantified data in (C) represent mean $\pm$ SEM. $n = 76$ cells for control siRNA, $n = 89$ cells for UBQLN4 siRNA#1, and $n = 81$ cells for UBQLN4 siRNA#2. P -values were calculated by Welch's t -test between the siRNA-transfected and the respective control condition ($*P < 0.01$).
- D** Depletion of UBQLN4 makes HeLa cells more sensitive to puromycin-induced cell death. After 48 h of UBQLN4 siRNA treatment, the cells were treated with 5 μ g/ml puromycin for 15.5 h, and then, viability was measured. The data represent mean $\pm$ SD calculated from three independent experiments ($n = 3$). P -values were calculated by Student's t -test compared with puromycin-treated control siRNA cells ($*P < 0.01$).

Figure 3. Δ SS mutant form of IL-2R α as a model for mislocalized proteins.

- A Schematic representation of the IL-2R α proteins used in this study. WT: wild type, Δ SS: signal sequence (SS)-deleted mutant, and Δ SS Δ TM: SS and transmembrane domain (TM)-deleted mutant.
- B IL-2R α Δ SS mislocalized to the cytosolic fraction. HeLa cells, expressing Flag-tagged IL-2R α WT or Δ SS, were fractionated to the cytosolic (Cyt.) and membrane (Mem.) fractions. Tubulin was used as a cytoplasmic marker, while calnexin was used for the ER membrane fraction.
- C IL-2R α Δ SS was not glycosylated. Flag-tagged IL-2R α WT or Δ SS proteins were expressed in HeLa cells and immunoprecipitated with an anti-Flag antibody. The precipitates were incubated with (+) or without (–) 10 units of the deglycosylation enzyme PNGase F for 2 h and subjected to Western blot analysis with an anti-Flag antibody. Glycosylated and non-glycosylated signals are indicated.
- D The glycosylated form of IL-2R α WT is a stable protein, while mislocalized IL-2R α Δ SS is degraded rapidly via its TMD. A series of IL-2R α deletion proteins were expressed in HeLa cells and then chased with 20 μ g/ml CHX for the indicated periods. Actin was used as a loading control. The right panel is a quantification graph of the left blot signals, and data represent mean $\pm$ SD calculated from four independent experiments ($n = 4$).
- E IL-2R α Δ SS is degraded in a proteasome-dependent manner. HeLa cells expressing Flag-tagged IL-2R α Δ SS were treated with 20 μ g/ml CHX as well as 0.1% DMSO (DM), 10 μ M MG-132 (MG), 2 μ M bortezomib (Bor), 10 μ M leupeptin (Leu), 0.1 μ M bafilomycin A1 (Baf), or 10 μ g/ml pepstatin A (pep. A)/E64d for the indicated periods. The right panel indicates the quantified data of the left blot signals presented as mean $\pm$ SD calculated from three independent experiments ($n = 3$).
- F The Flag-tagged IL-2R α Δ SS mutant and T7-tagged ubiquitin were co-expressed in HeLa cells, and the cells were treated with (+) or without (–) 10 μ M MG-132. After 4 h, Flag precipitates were blotted with anti-T7 and anti-Flag antibodies.
- G HeLa cells expressing Flag-tagged IL-2R α WT, Δ SS, and Δ SS Δ TM proteins were treated with (+) or without (–) 10 μ M MG-132. At 4 h after MG-132 treatment, the cells were lysed and analyzed by immunoblotting using the indicated antibodies. Note that a higher migrating form of non-glycosylated (and thus defective) IL-2R α WT (indicated by the arrowhead) is detected strongly in the presence of MG-132.

Source data are available online for this figure.

To demonstrate directly that IL-2R α Δ SS fails to assemble in the membrane, we examined its mislocalization to the cytosolic fraction. As shown in Fig 3B, wild-type IL-2R α (IL-2R α WT) was

detected in the membrane fraction (Mem), while the majority of IL-2R α Δ SS was present in the cytosolic soluble fraction (Cyt), even though it possesses a highly hydrophobic TMD. To investigate

further whether IL-2R α Δ SS fails to translocate to the ER membrane, we analyzed the glycosylation state of IL-2R α protein. In the case of IL-2R α WT expression, smear bands were detected (Fig 3C, indicated as glycosyl), and these signals were shifted down by treatment with PNGase F, a deglycosylation enzyme (Fig 3C, indicated as non-glycosyl). This result suggests that the majority of IL-2R α WT is indeed glycosylated and thus assembled in the ER membrane fraction. On the contrary, glycosyl modification was detected scarcely for the Δ SS form of mutant IL-2R α (Fig 3C), indicating that IL-2R α Δ SS is not inserted properly into the ER membrane.

A previous study suggested that IL-2R α Δ SS is a highly unstable protein [69], and we provide additional evidence for its ubiquitin-proteasome-mediated degradation (Figs 3D–F and EV2A and B, Δ SS). Indeed, the proteasome inhibitor bortezomib blocked its degradation as efficiently as MG-132-treatment, while both leupeptin and bafilomycin A1, lysosomal inhibitors, failed to stabilize IL-2R α Δ SS (Fig 3E). We also confirmed that the C-terminal TMD is responsible for the rapid cytosolic degradation of IL-2R α Δ SS (Fig 3D), and removal of the TMD from IL-2R α Δ SS largely abolished the MG-132-induced increase in its levels (Fig 3G). Thus, the exposed hydrophobic domain of misassembled IL-2R α in the cytosol functions as a major degradation signal for the ubiquitin-dependent degradation pathway. However, we do not exclude the possibility of the existence of another potential “degron sequence” in IL-2R α Δ SSATM, since residual effects of MG-132 were observed occasionally for IL-2R α Δ SSATM protein (Fig EV2C, Δ SSATM).

It should be noted that the levels of the non-glycosylated form of IL-2R α WT, a high-mobility band close to that of IL-2R α Δ SS, were sensitive to MG-132 (Fig 3G, arrowheads and Fig EV2D, non-glycosylated), as is the case for defective IL-2R α , IL-2R α Δ SS, although those of the low-mobility glycosylated forms of IL-2R α WT, representing mature folded ER species, were not (Fig 3G, glycosyl and Fig EV2D, glycosylated). These observations suggest that some portion of IL-2R α WT fails to translocate to the ER and thus remains in the cytosol as a defective protein. These results indicate that deleting the SS from IL-2R α , as well as naturally occurring defective assembly of TM proteins, leads to the production of client proteins for the ubiquitin-dependent degradation system. We suggest that IL-2R α Δ SS can be considered as an appropriate model for such newly synthesized (and rapidly degraded) cytosolically mislocalized TM proteins.

UBQLN4 is essential for misassembled membrane protein degradation

Identification of the mechanism for the targeted degradation of misassembled TM proteins is crucial for the quality control of newly synthesized TMD polypeptides. Hegde and colleagues reported that the mislocalized prion protein (PrP) is a client for the BAG6-mediated cytosolic degradation pathway [25,68]. In agreement with their findings for PrP, we found that BAG6 co-precipitated with the defective TM protein IL-2R α Δ SS (Fig EV3A). Furthermore, this interaction depended on the presence of a C-terminal TMD (Fig EV3A). In addition, knockdown of BAG6 blocked the proteasome-mediated degradation of IL-2R α Δ SS (Fig EV3B). Since BAG6 is now found to be critical for the degradation of IL-2R α Δ SS, we examined the possible participation of UBQLN4, a BAG6-binding

ubiquitin receptor protein associated with newly synthesized polyubiquitinated polypeptides (Figs 1 and EV1A), in the metabolism of the misassembled IL-2R α Δ SS model substrate. We designed three independent siRNA constructs for *UBQLN4* to avoid possible adventitious off-target effects of each double-stranded RNA and provided evidence that all three blocked the degradation of IL-2R α Δ SS effectively (Fig 4A and B, siRNA#1, #2, and #3). Furthermore, we showed that three scrambled double-stranded RNAs for each knockdown construct (Fig EV4A, siRNA#1scr, #2scr, and #3scr), in addition to the universal negative control siRNA construct (Fig 4A and B), did not affect IL-2R α Δ SS stability. These results suggest that endogenous UBQLN4 is indeed essential for IL-2R α Δ SS metabolism. It is interesting to note that *UBQLN4* knockdown did not abolish, but rather strengthened, the polyubiquitin modification of IL-2R α Δ SS (Fig 4C, compare lanes 1 and 3, Appendix Fig S3A), while *BAG6* knockdown abolished ubiquitination (Fig 4C, compare lanes 2 and 6, Appendix Fig S3A). These observations support the idea that UBQLN4 could be essential for the degradation of misassembled TM proteins, possibly at the post-ubiquitination step of IL-2R α Δ SS degradation.

An unembedded TMD is essential for UBQLN4 recognition

To reveal how UBQLN4 regulates the turnover of misassembled TM proteins, we examined its physical interaction with IL-2R α Δ SS. We immunoprecipitated a series of IL-2R α deletion mutants with full-length UBQLN4 and examined their interaction (Fig 5A). We found that UBQLN4 co-precipitated with IL-2R α Δ SS far more efficiently than with IL-2R α WT (Fig 5A, lanes 8 and 10). Furthermore, UBQLN4 associated with IL-2R α Δ SS even more under proteasome inhibition (Fig 5A, lanes 9 and 10). In addition, the TMD of IL-2R α Δ SS was found to be indispensable for UBQLN4 recognition, since IL-2R α Δ SSATM did not interact with UBQLN4 very much, even in the presence of MG-132 (Fig 5A, lane 12). Conversely, similar results were observed when we immunoprecipitated UBQLN4 (Fig 5B, Appendix Fig S3B); it co-precipitated IL-2R α Δ SS effectively, while it scarcely precipitated the TMD-deleted mutant (Fig 5B, Δ SSATM). Note that the glycosylated 48 kDa form of IL-2R α WT co-precipitated scarcely with UBQLN4 (Fig 5B). These observations suggest that UBQLN4 preferentially targets misassembled TM proteins for proteasome-mediated degradation through an exposed TMD-dependent mechanism.

The STI-II region of UBQLN4 is necessary for its recognition of unembedded IL-2R α

All UBQLN family proteins possess N-terminal UBL and C-terminal UBA domains (Fig 6A) [50–52,70–72]. The overall amino acid sequence homology between UBQLN1 and UBQLN4 is high (61%), suggesting they might share common functions. To determine whether UBQLN1 also recognizes misassembled TM proteins, we examined the affinity of UBQLN1 for IL-2R α Δ SS. Although UBQLN1 reportedly interacts with polyubiquitinated substrates and proteasomes [50], UBQLN1 poorly precipitated IL-2R α Δ SS substrates (and BAG6) compared to UBQLN4 (Figs 6B, E and F, and EV1A). Indeed, our quantified data show the clear preference of IL-2R α Δ SS binding for UBQLN4 compared to that for UBQLN1 (Fig EV4B), providing convincing evidence for their specificity.

Figure 4. UBQLN4 is essential for the degradation of mislocalized IL-2R α Δ SS.

A, B IL-2R α Δ SS is stabilized in *UBQLN4* knockdown cells. HeLa cells were transfected with three distinct siRNA duplexes for *UBQLN4* (*UBQLN4* siRNA#1–#3) or control siRNA. At 48 h after siRNA transfection, Flag-tagged IL-2R α Δ SS was expressed in the cells. At 24 h after IL-2R α Δ SS transfection, the cells were chased with 20 μ M CHX and harvested at the indicated time after CHX addition. Anti-Flag signals in the control or *UBQLN4* siRNA-treated cells (siRNA#1–#3) were quantified at the indicated time points. The data represent mean $\pm$ SEM calculated from four independent experiments ($n = 4$). The *UBQLN4* signal is marked by an arrowhead, while the band just below it (indicated by an asterisk) is a non-specific band, since this signal was never affected by any of the *UBQLN4* siRNAs. Actin was used as a loading control.

C Polyubiquitin modification of IL-2R α Δ SS was not diminished, but rather strengthened, in *UBQLN4* knockdown cells. At 48 h after transfection of HeLa cells with siRNA for *UBQLN4* or *BAG6*, Flag-tagged IL-2R α Δ SS and T7-tagged ubiquitin were expressed with (+) or without (–) 10 μ M MG-132. From these cells, Flag-tagged IL-2R α Δ SS was affinity-purified using hot lysis analysis, and Flag precipitates were blotted with an anti-T7 antibody to detect polyubiquitination of IL-2R α Δ SS. The *UBQLN4* signal is marked by an arrowhead, and the asterisk indicates a non-specific band. Actin was used as a loading control.

Source data are available online for this figure.

In order to obtain insights into the differences between UBQLN family proteins, we performed comprehensive disorder/order structural prediction analysis. When the amino acid sequences of UBQLN1 and UBQLN4 were analyzed using the secondary structure prediction program PONDR-FIT, their overall disorder/order tendency was nearly identical. For example, the UBL and UBA domains of UBQLN1 and UBQLN4 had a very low disorder score (Fig 6C, black boxes), exactly matching the fixed structures of these domains as determined by X-ray crystal diffraction analysis (PDB ID codes 2KNZ for UBQLN4 UBA, 2JY5 for UBQLN1 UBA and 2KLC for UBQLN1 UBL). Both UBQLN family proteins contain putative STI1-like motifs between the UBL and UBA domains, and we designated two tandem STI1 repeats in the N-terminal half as STI-I, and two other tandem repeats in the C-terminal half as STI-II (Fig 6A). While

STI-I (amino acids 182–251 and 192–261 for human UBQLN1 and UBQLN4, respectively) had a high disorder score in all UBQLN family members (Fig 6C, green boxes), we found that the STI-II sequence of UBQLN4 (amino acids 393–476 for human UBQLN4) had the characteristic lowest disorder score (Fig 6C, red box), making a clear contrast with the high disorder score of STI-II in UBQLN1 (Fig 6C, blue box) and UBQLN2. Similar results were obtained using the structural prediction algorithms RONN and Displot, with the STI-II region demonstrating the lowest disorder tendency in UBQLN4. The structured feature of UBQLN4 STI-II is evolutionarily conserved from Amphibia, Reptilia, and Aves to their mammalian orthologs (Appendix Fig S2). These analyses suggest that the STI-II region of UBQLN4 forms a unique, distinct, and evolutionarily conserved structural domain.

Figure 5. UBQLN4 recognizes misassembled IL-2Rα through its unembedded TMD.

A A series of Flag-tagged truncated mutants of IL-2Rα substrates were expressed in HeLa cells with T7-tagged UBQLN4 and treated with (+) or without (–) 10 μM MG-132 for 4 h. Flag-IL-2Rα substrates were immunoprecipitated, and their co-precipitation with UBQLN4 was analyzed.

B T7-UBQLN4 was immunoprecipitated, and its interactions with a series of IL-2Rα proteins were detected.

Source data are available online for this figure.

When we compared the sequences of the STI-II regions of UBQLN1 and UBQLN4, they are highly conserved with only 10 residue characteristic substitutions with increased hydrophobicity (Fig 6D). To investigate whether the structural and sequential features of the UBQLN4 STI-II domain might be linked with its functional properties, we prepared a mutant protein in which UBQLN4-type STI-II was replaced with UBQLN1-type STI-II (Fig 6A and E). We found that such a substituted mutant, designated as 4-STI-II-1, showed a greatly reduced affinity for IL-2Rα ΔSS when compared to WT UBQLN4 (Fig 6E). When STI-II was transferred from UBQLN4 into UBQLN1, this reverse chimera (designated 1-STI-II-4) failed to

stimulate binding with IL-2Rα ΔSS (Fig 6F), suggesting that STI-II is not the sole determinant of binding to specific substrates. In agreement with this view, a recombinant STI-II fragment fused with GST failed to associate with IL-2Rα ΔSS, while recombinant GST-UBQLN4 WT co-precipitated with this client protein *in vitro* (Fig EV4C).

To determine whether the STI-II domain is indeed required for IL-2Rα ΔSS recognition by UBQLN4, we prepared a series of UBQLN4 deletion mutants (Fig 7A). While deletion of either the UBL or STI-I region of UBQLN4 did not affect its affinity for IL-2Rα ΔSS (Fig 7B, ΔUBL and ΔSTI-I, respectively), we found that truncation of the STI-II domain, as well as the C-terminal UBA domain, greatly compromised its affinity for IL-2Rα ΔSS (Fig 7B, ΔSTI-II and ΔUBA, respectively). Furthermore, we provide evidence that the C-terminal half of the UBQLN4 fragment (from STI-II to the C-terminal UBA domain, residues 378–596) sufficiently co-precipitated IL-2Rα ΔSS substrates, while its STI-II-truncated mutant (residues 471–596) showed reduced affinity (Fig 7C). These results suggest that the STI-II region might be necessary, but not sufficient, for substrate recognition in UBQLN4 (Fig 7B and C). It is also apparent that UBL domain is dispensable for substrate binding.

The STI-II domain of UBQLN4 is essential for endogenous client recognition

Since both the UBA and STI-II domains of UBQLN4 are essential for its binding with the misassembled model substrate protein IL-2Rα ΔSS (Fig 7B), which can be polyubiquitinated for proteasomal degradation (Fig 3F), we examined whether deletion of these regions affects its co-precipitation with not only the defective model protein but also with endogenous newly synthesized polyubiquitinated substrates (Fig 1D). We immunoprecipitated a series of UBQLN4 deletion mutants from MG-132-treated cell extracts, and the precipitates were probed with an anti-T7-ubiquitin antibody (Fig 7D, Appendix Fig S3C). It is quite natural that deletion of the UBA domain results in a reduction of polyubiquitinated proteasomal substrate co-precipitation (Fig 7D, ΔUBA), since the UBA domain is known to recognize the polyubiquitin moiety [58,60–62]. Our notable finding is that deletion of the STI-II region also compromised co-precipitation of polyubiquitinated client proteins from the cell extracts as effectively as UBA domain truncation (Fig 7D, ΔSTI-II), suggesting that the STI-II domain of UBQLN4 possesses an essential function for substrate recognition. It is also apparent that the UBA domain itself is not sufficient to recruit endogenous

Figure 6. The STI-II region of UBQLN4 determines its specificity to misassembled IL-2Rα.

A Schematic representation of the UBQLN1, UBQLN4, and STI-II mutant proteins used in this study.

B UBQLN4 dominantly co-precipitates IL-2Rα ΔSS over UBQLN1. Precipitates of Flag-tagged UBQLN1 or UBQLN4 from HeLa cell lysates were probed with IL-2Rα ΔSS.

C The complete amino acid sequences of human UBQLN1 and UBQLN4 were analyzed using the disorder/order structure prediction program PONDR-FIT. The STI-II region of UBQLN4 is indicated by the red box, and that of UBQLN1 is boxed blue. Note that the disorder tendency of the UBL, STI-I, and UBA regions is nearly identical between UBQLN1 and UBQLN4 (indicated by black and green boxes), while that of the STI-II region is quite distinct, and the STI-II of UBQLN4 shows a high probability of an ordered structure. The numbers denote the corresponding amino acid positions in these proteins.

D Amino acid sequence alignments of the STI-II regions of UBQLN1 and UBQLN4. UBQLN4-specific residues are shown as red characters. HS: *Homo sapiens* (Mammalia), MM: *Mus musculus* (Mammalia), GG: *Gallus gallus* (Aves), PS: *Pelodiscus sinensis* (Reptilia), and XL: *Xenopus laevis* (Amphibia).

E Substitution of the STI-II sequence of UBQLN4 with that of UBQLN1 (designated as the 4-STI-II-1 mutant protein) abolished its high affinity to the IL-2Rα ΔSS client protein.

F Substitution of the STI-II sequence of UBQLN1 with that of UBQLN4 (designated as the 1-STI-II-4) did not enable UBQLN1 to recognize IL-2Rα ΔSS.

Source data are available online for this figure.

Figure 6.

Figure 7. STI-II region of UBQLN4 is essential for its binding to client proteins.

A Schematic representation of the UBQLN4 deletion mutant proteins used in this study.

B–D The wild-type (WT) form of Flag-tagged UBQLN4 and its truncated derivatives were expressed in HeLa cells with T7-tagged IL-2R α Δ SS (B and C) and T7-tagged ubiquitin (D). At 4 h after the addition of 10 μ M MG-132, UBQLN4 was immunoprecipitated with an anti-Flag antibody and the precipitates were blotted with an anti-T7 antibody.

E GST-tagged and purified UBQLN4 and its truncated derivatives (Δ UBA and Δ STI-II) were incubated with K48-linked polyubiquitin chains. After *in vitro* GST pull-down, the precipitates were probed with an anti-polyubiquitin (FK2) antibody.

Source data are available online for this figure.

polyubiquitinated substrates in the case of UBQLN4. It is of note that deletion of STI-II did not affect the direct polyubiquitin chain recognition ability of the C-terminal UBA domain, since our *in vitro* experiments showed that the STI-II-deleted form of purified UBQLN4 protein bound to a K48-linked polyubiquitin chain as efficiently as to the WT protein (Fig 7E). These observations suggest that the ubiquitin-binding ability of the UBQLN4 UBA domain itself is not sufficient to recognize newly synthesized client proteins. Collaboration between the UBA and STI-II domains of UBQLN4 might be necessary for the efficient recognition of misassembled TM protein substrates.

Endogenous substrates of SRP are clients of UBQLN4

Successful biogenesis of TM proteins is mediated through the SRP, which recognizes the SS of client TM proteins to bring them to the SRP receptor on the rough ER [6–8,13–15,22]. Since we

identified the Δ SS truncated form of IL-2R α as a client of UBQLN4, we examined whether depletion of the SRP results in the production of defective cytoplasmic species derived from WT IL-2R α . As shown in Fig 8A, knockdown of SRP54, the SS-binding subunit of the SRP, indeed led to a reduction of the glycosylated form of mature IL-2R α (Fig 8A, lanes 3 and 4, indicated as assembled). Furthermore, SRP54 depletion induced the accumulation of the non-glycosylated form of IL-2R α WT in the presence of MG-132 (Fig 8A, lane 4, indicated as defective), similar to the case for the truncation of its SS (Fig 3C), suggesting that cytosolically mislocalized TM proteins can be induced by defects in the SRP. Importantly, the non-glycosylated form of IL-2R α WT in SRP-suppressed cells was recognized efficiently by UBQLN4 (Fig 8A, lane 8, indicated by an arrowhead). These observations suggest that UBQLN4 contributes to the quality control of misassembled cytoplasmic TM proteins under SRP defective conditions, as for IL-2R α SS.

In the condition of *SRP54* knockdown, it is probable that a number of endogenous TMD proteins with an N-terminal SS also fail to assemble in the ER and thus accumulate as polyubiquitinated defective TM proteins. Since UBQLN4 associates with a model misassembled TM protein (Fig 5) as well as endogenous polyubiquitinated newly synthesized polypeptides (Fig 1B and D) through a common STI-II region (Fig 7B–D), we reasoned that not only IL-2R α but also a variety of endogenous defective TM proteins might be general clients of UBQLN4. To investigate whether UBQLN4 might be involved with a variety of SRP substrates, we inactivated *SRP54* using siRNA. As shown in Fig 8B, *SRP54* knockdown resulted in a slight accumulation of polyubiquitinated proteins, even in the absence of MG-132, and we found an increased level of UBQLN4-associated polyubiquitinated species that were induced by *SRP54* knockdown (Fig 8B, Appendix Fig S3D). In the presence of MG-132, *SRP54* siRNA further stimulated the association of polyubiquitinated substrates with UBQLN4 (Fig 8C, IP lane, with MG-132). These results suggest that at least a part of the endogenous pool of SRP substrates became a client of UBQLN4 for targeted degradation when the function of the SRP was compromised.

Figure 8. SRP deficiency enhances the interaction between UBQLN4 and its client proteins.

A UBQLN4 interacts with non-glycosylated (and thus defective) IL-2R α WT in *SRP54* knockdown cells. After treatment of HeLa cells with *SRP54* siRNA (10 nM) for 48 h, Flag-tagged IL-2R α WT and T7-tagged UBQLN4 proteins were expressed, and the cells were treated with (+) or without (–) 10 μ M MG-132 at 4 h before harvesting. UBQLN4 was affinity-purified from cell extracts and probed with an anti-Flag antibody. Note that the levels of non-glycosylated IL-2R α WT (indicated as defective) increased, while the glycosylated form of this protein (indicated as assembled) decreased in *SRP54* knockdown cells.

B, C *SRP54* knockdown stimulates polyubiquitinated proteins co-precipitation of UBQLN4. Flag-tagged UBQLN4 and T7-tagged ubiquitin were expressed in *SRP54* siRNA-treated cells, and Flag precipitates were probed with an anti-T7 antibody to detect polyubiquitinated client co-precipitation with UBQLN4 under no addition of MG-132 (**B**). Note that the interaction of UBQLN4 with polyubiquitinated substrates was further increased in *SRP54* knockdown cells treated with MG-132 for 4 h before harvesting (**C**).

Source data are available online for this figure.

Discussion

The majority of nascent TMD proteins are integrated into the ER membrane by virtue of an SS- and SRP-mediated co-translational process [6,8,9,12,14]. In accordance with this notion, we showed that SS truncation or SRP suppression resulted in the production of a defective form of IL-2R α . With such a defective protein model, we identified the UBL-UBA ubiquitin receptor UBQLN4 as a binding protein whose function is closely linked with the metabolism of newly synthesized and misassembled TMD polypeptides in the cytoplasm.

Accumulating evidence suggests that the substrates of both the co- and post-translational ER targeting pathways are polyubiquitinated in the aqueous cytosol in a BAG6-dependent manner when targeting fails [25,43,44,47,68]. Mammalian PrP also showed preferential stabilization as a non-glycosylated precursor species when BAG6 was knocked down [25]. In the present study, we found that UBQLN4 is included in the BAG6 complex, while other UBQLN4 paralog family proteins were found to have a much weaker cellular interaction with BAG6 (Fig EV1A). This observation suggests that BAG6 collaborates preferentially with UBQLN4 and their function might be closely related. Indeed, both BAG6 [25] and UBQLN4 (this study) were shown to be essential for targeting improperly assembled TM proteins for proteasomal degradation. However, the function of UBQLN4 is not identical to that of BAG6, because the knockdown phenotype of *UBQLN4* with polyubiquitinated protein accumulation was the opposite of that of *BAG6*. Indeed, *UBQLN4* knockdown stimulated the cellular accumulation of polyubiquitinated IL-2R α Δ SS, while *BAG6* knockdown reduced it (Fig 4C). Thus, they might play partly distinct roles in their complex.

How UBQLN4 recognizes client substrates is an important issue to understand the quality control mechanism of newly synthesized TM proteins. UBQLN4 possesses two tandem repeats of the STI1 motif in its central region [49,73,74]. STI-I motifs and their upstream region in the N-terminal half of UBQLN4 are involved in SS recognition of various ER luminal proteins [51] as well as in the interaction with LC3, an autophagic regulator [74], while the functions of the STI-II region in UBQLN4 remain completely elusive to date. In this study, we found that the STI-II region of UBQLN4 is necessary, but

not sufficient, for the recruitment of mislocalized TMD proteins (Fig 7B). We also showed that the STI-II region of UBQLN4 is critical for co-precipitation with newly synthesized polyubiquitinated proteins (Fig 7D). While all UBQLN family proteins possess an STI-II motif, UBQLN1 associates with neither IL-2R α Δ SS (Fig 6B, E and F) nor BAG6 (Fig EV1A), in contrast to UBQLN4. These observations suggest that the STI-II region of UBQLN4 is a distinct domain, as suggested by disorder/order prediction analysis (Fig 6C), which plays a key role in the discrimination of unassembled TM proteins.

Groettrup *et al* [23] reported that an MHC class I-restricted peptide, mainly derived from the degradation products of newly synthesized defective polypeptides [66,75–78], contains the cleavage site of the ER signal peptidase [23]. As signal peptides for ER targeting are cleaved off immediately after their co-translational insertion into the lumen of the ER [8], this uncleaved antigenic peptide did not originate from its retro-translocation from the ER [23] and thus could not be derived from ER-associated protein degradation (ERAD), a major TM protein quality control machinery in eukaryotic cells [77,79–85]. Instead, this endogenous antigenic protein could be degraded by cytoplasmic proteasomes before it reaches the lumen of the ER [23]. In accordance with this idea, ablating the SS of a type I membrane protein reportedly results in its default delivery to the cytosol and accelerated turnover for MHC-mediated antigen presentation, in contrast to the substantially delayed presentation from the ERAD-derived counterpart pathway [69,86]. Although we do not exclude the possibility that a portion of IL-2R α Δ SS is an ERAD substrate, this seems largely unlikely since the typical ERAD pathway is effective only after proteins are incorporated into the ER membrane. It should be noted that UBQLN4 also recognizes the non-glycosylated (and thus mislocalized) form of wild-type IL-2R α that possesses a non-processed SS (with a T7-tag) at its N-terminus in the cytoplasm (Fig EV5). These observations also support our hypothesis that UBQLN4 eliminates MLP via a non-ERAD pathway. It was reported that the cytosolic face of the ER membrane serves as a “platform” for the rapid degradation of cytosolic substrates that exposed hydrophobic [87], and ERAD-C components are responsible for such “non-ERAD” protein degradation [87,88]. We speculate that the ERAD-C machinery might also be utilized for defective IL-2R α degradation on the cytosolic surface of the ER. An interesting challenge for the future will be to determine how the UBQLN4 ubiquitin receptor collaborates with chaperones/E3 ligases/BAG6/ERAD-C machineries. Clarification of the linkage between defects in the UBQLN4-BAG6 complex and various human diseases such as immune disorders, neurodegeneration, and diabetes should also be a relevant prospect for future extensive investigations, since the quality control system for MLP is intimately linked with these diseases. Our findings in this study will provide important clues to reveal these critical points.

Materials and Methods

Plasmid construction

Full-length cDNAs of UBQLN4 (*Mus musculus* and *Homo sapiens*), ubiquitin (*Mus musculus*) and those of UBQLN1 and UBQLN3 (*Homo sapiens*) were amplified by PCR from cDNA libraries derived from HeLa and NIH3T3 cells, respectively. The IL-2R α cDNA was amplified from mouse thymic cDNA library. The PCR fragments

were cloned into pCI-neo-based mammalian expression vectors (Promega) with N-terminal 3xFlag- or 3xT7-tags with their products. Truncated and mutated versions of UBQLN4 and IL-2R α were prepared by PCR. Vectors were used for experiments after verification of the sequence of inserted DNA.

Mammalian cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Wako) supplemented with 10% heat-inactivated calf serum at 37°C under 5% CO₂ atmosphere. Transfections of the expression vectors were performed with HilyMax (Dojindo, Japan) or polyethylenimine “MAX” transfection reagent (Polysciences, Inc.) according to the protocols supplied by the manufacturers. At 18–24 h after transfection, the cells were harvested and subjected to immunological analysis unless otherwise noted.

RNA interference

For knockdown analysis of UBQLN4, three independent duplex siRNAs covering the targeted sequences

5'-CAAACAGCAGGGUGACUUUtt-3' (UBQLN4 siRNA#1),
5'-CUCAAUAACCCUGAACUCAtt-3' (UBQLN4 siRNA#2),
5'-CUCUUCAGAUUGCUGGCAGUtt-3' (UBQLN4 siRNA#3).

were synthesized (SIGMA Genosys). UBQLN4 siRNA#1 was designed to target the 3'-untranslated region of UBQLN4 where the sequence is quite diverged in all UBQLN family genes. The other two siRNA sequences were also designed to avoid matching higher sequence conserved regions within UBQLN family genes. We also synthesized three scrambled duplex siRNA sequences as negative controls to the above UBQLN4 knockdown sequences, respectively,

5'-GAUGAAACAUAGCGUCCGUtt-3' (UBQLN4 siRNA#1scr),
5'-AACACAAAUCCGCACUCUUtt-3' (UBQLN4 siRNA#2scr),
5'-UUCUUAUUGUACCGGGGCGtt-3' (UBQLN4 siRNA#3scr).

In addition, MISSION siRNA Universal Negative Control 1 (Sigma-Aldrich) was used as a general negative control in every experiment. The duplex siRNA sequences of UBQLN1 and UBQLN2 are 5'-GGTGTCTGGCGCCCCGCGGCCG-3' and 5'-CAUGUACACUGA CAUUCAAtt-3', respectively. For SRP54 depletion, the duplex siRNA sequence 5'-CACUUAUAGAGAAGUUGAAAtt-3' was synthesized. BAG6 depletion was performed as described previously [32] with two independent duplex siRNA sequences of

5'-UUUCUCCAAGAGCAGUUUAtt-3' (BAG6 siRNA#1).
5'-CAGAAUGGGUCCCUAUUAUtt-3' (BAG6 siRNA#2).

Transfections of HeLa cells with duplex siRNA were performed using Lipofectamine 2000 (Invitrogen) according to the protocol provided by the manufacturer. The efficacy of each siRNA was verified by immunoblot with their specific antibodies listed in the next section.

Immunological analysis

The anti-UBQLN4 antibody was prepared for this study as follows. Bacterially produced full-length *Mus musculus* UBQLN4 was mixed and emulsified with TiterMax Gold (TiterMax USA, Inc.) and then

inoculated into a rabbit with 2-week intervals. After 2 weeks from the third inoculation, anti-UBQLN4 IgG in the serum was affinity-purified with Protein A-Sepharose (GE Healthcare). We confirmed that the endogenous immunosignal of this antibody in HeLa cells was suppressed by three independent *UBQLN4* siRNA duplex (*UBQLN4* siRNA#1, #2, and #3).

For immunoprecipitation analysis, HeLa cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with immunoprecipitation (IP) buffer containing 20 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 10 mM N-ethylmaleimide, 25 μ M MG-132, and protease inhibitor cocktail (Nacalai tesque). In the case of Figs 3C and 5A, HeLa cells were lysed with chaperone buffer containing 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Tween-20, 10% glycerol, 10 mM N-ethylmaleimide, 25 μ M MG-132, and protease inhibitor cocktail. The lysates were sonicated (except for Fig 8B), centrifuged at 20,630 $\times$ g for 20–30 min at 4°C, and mixed with 4–10 μ l of anti-Flag M2 affinity gel (Sigma) or anti-T7 tag antibody agarose (Novagen) for 10 min to 2 h at 4°C. After the beads had been washed five times with the IP buffer or chaperone buffer, the immunocomplexes were eluted by SDS sample buffer.

For hot lysis analysis, HeLa cells were washed by PBS and lysed with hot lysis buffer containing 1% SDS, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 25 μ M MG-132, 10 mM N-ethylmaleimide, and inhibitor cocktail. The lysates were heated in 90°C for 15 min and sonicated. After the lysates were centrifuged at 20,630 $\times$ g for 20 min at room temperature, the supernatant was diluted for fourfold with buffer A (containing 1% Triton X-100, 50 mM Tris-HCl pH 7.5, and 150 mM NaCl) and mixed with 5 μ l anti-Flag M2 affinity gel for 10 min at 4°C. After the beads had been washed five times with buffer A, the immunocomplexes were eluted by SDS sample buffer.

For Western blot analyses, whole-cell lysates and the immunoprecipitates were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride transfer membrane (GE Healthcare, Pall Corporation). The membranes were then immunoblotted with specific antibodies as indicated and then incubated with horseradish peroxidase-conjugated antibody against mouse or rabbit immunoglobulin (GE Healthcare), followed by detection with ECL Western blotting detection reagents (GE Healthcare), Clarity™ Western ECL Substrate (Bio-RAD), or Immobilon Western chemiluminescent HRP Substrate (Merck Millipore).

The following antibodies were used in this study: anti-UBQLN4 (prepared in this study), anti-BAG6 [32], anti-polyubiquitin FK2 (MBL or Nippon Bio-Test Laboratories Inc.), anti-SRP54 (BD Biosciences), anti-UBQLN1 (Lifespan Biosciences, Inc.), anti-UBQLN2 (Abnova), anti-Rpt5 (Enzo Life Sciences), anti-Flag M2 monoclonal (Sigma), anti-Flag polyclonal (Sigma), anti-T7-tag monoclonal (Novagen), anti- β -actin (Sigma), anti-calnexin (Sigma), anti-tubulin (Santa Cruz Biotech.), and anti-GST (Santa Cruz Biotech.).

Subcellular fractionation

The expression vectors of IL-2R α WT with N-terminal Flag-tags and its Δ SS derivative were transfected into HeLa cells. After 22 h of transfection, subcellular fractionation was performed using a EzSubcell Extract (ATTO) according to the protocol provided by the manufacturer.

Microscopic observations

For immunocytochemical observations, HeLa cells that grown on micro-cover glass (Matsunami) were washed twice with PBS, fixed by incubating in 4% paraformaldehyde for 20 min at 4°C, and were permeabilized with 0.1% Triton X-100 for 3 min in room temperature. Fixed cells were blocked with 3% heat-inactivated calf serum in PBS and then reacted with a series of primary antibodies at room temperature for 1 h (Fig 2A and B). Alexa Fluor^R 488-conjugated anti-mouse IgG, Alexa Fluor 594-conjugated anti-mouse IgG, and Alexa Fluor 488-conjugated anti-rabbit IgG antibodies (Invitrogen) were used as secondary antibodies at dilutions of 1:500. To observe the nucleus, cells were stained with Hoechst 33342. Immunofluorescent images were obtained with BIOREVO BZ9000 fluorescence microscope (Keyence, Japan). BZ-II Analyzer application (Keyence, Japan) was used to quantify the number of ALIS. For the quantification of ALIS formation, polyubiquitin-positive dots with a luminance value > 150 were regarded as ALIS.

GST pull-down and ubiquitin-binding assay

For the expression of GST-fused recombinant proteins, cDNAs encoding *Mus musculus* UBQLN4, as well as their truncated derivatives, were subcloned into pGEX6P1 expression vector (GE Healthcare). GST-fused proteins were expressed in *E. coli* BL21 (DE3) by adding 0.1 mM IPTG. At 4 h from IPTG addition, *E. coli* were harvested and lysed with PBS-T (0.1% Tween-20 in PBS) and sonicated. The lysates were centrifuged at 20,630 $\times$ g for 20 min at 4°C. The supernatant was mixed with 10 μ l glutathione-immobilized beads (GE Healthcare) for 10–30 min at 4°C. After the beads had been washed three times with PBS-T, the beads were mixed with 3 μ g K48-linked polyubiquitin chains (R&D Systems, Inc.), incubated at 4°C for 30 min, and washed three times with PBS-T. Polyubiquitin chain co-precipitations were analyzed by Western blot analysis with anti-ubiquitin (FK2) and anti-GST antibodies. In the case of Fig EV4C, the beads with purified GST-UBQLN4 or those with GST-STI-II were mixed with HeLa cells extracts that expressing Flag-tagged IL-2R α Δ SS for 30 min at 4°C. After the beads had been washed three times with the IP buffer, the immunocomplexes were eluted by SDS sample buffer.

Cell viability assay

After 48 h of *UBQLN4*-specific siRNA treatment, HeLa cells were treated with 5 μ g/ml puromycin for the indicated time. Cell viability was determined using a Cell Counting kit 8 (Dojindo) according to the protocol provided by the manufacturer.

Statistical analysis

Evaluation of data was performed either by Welch's *t*-test or Student's *t*-test as indicated. All data in the figures are presented as the mean $\pm$ SEM or SD. *P*-value of < 0.05 is considered statistically significant.

Expanded View for this article is available online.

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Author contributions

RS and HK conceived and designed the experiments. RS performed all the experiments. RS and HK wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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