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Identification of ERAD components essential for dislocation of the null Hong Kong variant of α-1-antitrypsin (NHK)

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ABSTRACT

Misfolded proteins or orphan subunits of protein complexes are removed from the endoplasmic reticulum (ER) by ER-associated degradation (ERAD). ERAD requires dislocation, also known as retrotranslocation, of those unwanted proteins from the ER lumen to the cytosol for destruction by the proteasomes. Over one hundred ERAD component proteins have been identified but their role in dislocation remain poorly understood. Here we assessed the requirement of ERAD components for dislocation of NHK in live cells using our recently developed dislocation-induced reconstituted GFP (drGFP) assay. RNAi revealed that 12 out of 21 ERAD components examined are required for efficient dislocation of NHK among which Hrd1, Sel1L, GRP94 and p97/VCP are critically required. In addition, knockdown of 7 of the 21 components enhanced NHK dislocation. This study uncovers a complex functional network of proteins required for NHK dislocation.

1. Introduction

About one third of total cellular proteins enter the endoplasmic reticulum (ER) where they acquire their native conformation for function in the secretory pathway or extracellular space [1]. ER protein quality control mechanism monitors the protein folding states in the ER and removes terminally misfolded proteins and orphan subunits of protein complexes through proteasomal degradation, a process known as ER-associated degradation (ERAD) [2]. ER protein misfolding occurs during synthesis, due to genetic mutations or damage by environmental stresses [3]. Thus, ERAD is frequently challenged by a very diverse array of unwanted proteins.

ERAD substrates are either totally (luminal proteins) or partially (membrane-spanning proteins) enclosed by the ER membrane, but their destruction takes place in the cytosol by the proteasomes [4]. Thus, transport of the substrates across the ER membrane to the cytosol, known as dislocation or retrotranslocation, is a key step in ERAD. It is well established that ER membrane-anchored protein complex centered on an E3 ubiquitin ligase integrates identification, dislocation, ubiquitination, and proteasomal degradation of unwanted ER proteins during ERAD [5,6]. Over a dozen of E3 ubiquitin ligases have now been found to function in ERAD [5–11]. Many proteins associated with ERAD E3 ubiquitin ligases have been identified [12], suggesting a mechanistic complexity of the ERAD process. The complexity of ERAD machinery could be so evolved to meet the challenge of the vast array of unwanted proteins in order to preserve the function of the secretory pathway. However, dislocation of luminal substrates has been reconstituted *in vitro* using only a few purified components from *Saccharomyces cerevisiae* in which the ubiquitin ligase Hrd1p is the only required membrane protein [13]. Therefore, it is important to determine to what extent over one hundred ERAD component proteins identified so far are involved in dislocation in cells.

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Recently, we developed a dislocation-induced reconstituted GFP (drGFP) reporter for visualization and rapid quantification of protein dislocation during ERAD in live cells [14]. Taking advantage of this convenient assay in combination with RNAi and immunoblotting, we screened 21 previously isolated ERAD component proteins for their effects on dislocation of the luminal substrate, the null Hong Kong variant of α-1-antitrypsin (NHK). The results showed that most of these proteins screened regulate NHK dislocation, suggesting that dislocation requires a concerted action of a subset of proteins in the ERAD complex.

2. Materials and methods

2.1. Antibodies

Monoclonal anti-gp78 (2G5), polyclonal anti-SVIP, anti-Derlin-1 and anti-Hrd1 antibodies have been previously described [3,15,16]. Mouse monoclonal anti-β-actin (AC-74), anti-HA (HA-7), and rabbit polyclonal anti-Sel1L, anti-Aup1, anti-XTP3-B, anti-Derlin2, anti-Derlin3, and anti-VIMP antibodies were purchased from Sigma. Mouse monoclonal anti-HERP (19-Y), anti-UBE2J1 (B-6), anti-UBE2G2 (2E6), anti-UBXD8 (F-7), anti-Npl4 (D-1), anti-GRP94 (H-10), rabbit polyclonal anti-importin β (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc.. Mouse monoclonal anti-p97/VCP, rabbit polyclonal anti-Ufd1, anti-UBAC2 and rabbit monoclonal anti-OS9 antibodies were purchased from Affinity BioReagents, Bethyl Laboratories, Abgent and Abcam, respectively.

2.2. siRNAs

Silencer® Negative Control siRNA #1, p97/VCP (siRNA ID: 214796, sense sequence: GGGCACAUGUGAUUGUUAU), Ufd1 (siRNA ID: 139548, sense sequence: CCAACUCAGCCGACUUAAC), Npl4 (siRNA ID: 121816, sense sequence: GGACUAUCUAAACCAUCUC), gp78 (siRNA ID: 110866, sense sequence: CGCUCAGUUGAAAUAACAA), Hrd1 (siRNA ID: 44536, sense sequence: GGCCUUUGUCCUUGUCUUC), Sel1L (siRNA ID: 121516, sense sequence: GCAUAUCGGUAUCUCCAAA), SVIP (sense sequence: GACAAAAAGAGGCUGCAUC), VIMP (sense sequence: ACGGAAAUCGGACAGAAAG), importin β (sense sequence: GAGGUGGCUUGCUAUUGAU), GRP94 (sense sequence: GGAUCAAGGACGGAUGAUG), OS9 (sense sequence: GUACAAACAGCGCUAUGAG), XTP3-B (sense sequence: AGCAGUUGUUCCUACAGAA), UBE2G2 (sense sequence: UGACGAAAGUGGAGCUAAC), and Derlin-1 (sense sequence: UGGAUAUGCAGUUGCUGAU) siRNAs were ordered from Ambion (invitrogen) [6,7,14–18]. siRNAs for HERP, UBXD8, UBAC2, UBE2J1, Aup1, Derlin2 and Derlin3 were purchased from Santa Cruz Biotechnology, Inc..

2.3. Cell culture

HeLa cells stably expressing SP-S11-NHK-HA and S1-10 were established previously [14] and were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (V/V) fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin and Glutamine under 5% CO_2 in a humidified incubator.

2.4. Live cell imaging

Live cell images were acquired every 10 min under a 63× objective lens mounted on a Zeiss AxioObserver Z1 fluorescence microscope equipped with a high-sensitivity CCD camera (QuantEM 512SC; Photometrics, Tucson, AZ), environment control units and a Definitive Focus module.

2.5. RNA interference

HeLa cells stably expressing SP-S11-NHK-HA and S1-10 were transfected with control siRNA or siRNA targets to an ERAD component as indicated using lipofectamine RNAiMAX (Invitrogen). 72 h after transfection, cells were processed for Western blotting or drGFP measurement.

2.6. drGFP quantification

To measure the drGFP accumulated in siRNA-transfected cells, the cells were first treated with lactacystin (10 μM) for 4 h. After briefly washing with PBS, the drGFP (Ex = 488 nm, Em = 525 nm) intensity from 3×10^4 cells of each sample was measured with TECAN Infinite 200 Pro multifunctional microplate reader. Background fluorescence was measured using same amount of non-treated HeLa cells and subtracted from each sample reading. The relative fluorescence units (RFU) obtained for drGFP of control siRNA-transfected cells is arbitrary set as 1. The data are presented as the means+/− SD from 4 replicates. To take into account the NHK protein level differences between samples, the data are further normalized with the relative band densities of NHK in the Western blot.

2.7. Immunofluorescence

HeLa cells stably expressing SP-S11-NHK-HA and S1-10 were transfected with pcDNA3.1-Hrd1C1A [10]. 24 h after transfection, the cells were treated with lactacystin (10 μM) for 4 h. Then the cells were fixed in 2% paraformaldehyde for 10 min and permeabilized in 0.25% Triton X-100 for 3 min. The cells were then blocked in 5% BSA and incubated with anti-Hrd1 antibodies for 1 h followed by labeling with Alexa® Fluor 594 conjugated goat anti rabbit IgG (H + L) for 1 h.

3. Results

We used a previously reported split GFP system [19] to establish drGFP reporter for dislocation of NHK [14]: the last β strand (S11) of an engineered GFP was inserted after the signal peptide of NHK; the remaining portion, the number 1-10 β strands, of GFP (S1-10) was expressed in the cytosol of cells. When S11-tagged NHK is dislocated from the ER lumen to the cytosol, the S11 tag will automatically re-combine with the cytosolic S1-10, leading to reconstitution of GFP fluorescence (Fig. 1A, diagram). Accumulation of the dislocation-induced reconstituted GFP (drGFP) requires the inhibition of proteasome activity (Fig. 1A). We have previously generated HeLa cells that stably express both S11-NHK and S1-10 [11]. We demonstrated that treatment with proteasome inhibitor lactacystin caused a time-dependent accumulation of GFP fluorescence in HeLa cells as revealed by time-lapse imaging at 10 min interval for up to 4 h (Fig. 1B). Previous studies have reported that substrate ubiquitination is a key process required for dislocation as analyzed by subcellular fraction and *in vitro* reconstitution [20,21], but it has never been demonstrated in intact cells. NHK is a substrate of the ERAD E3 ubiquitin ligase Hrd1. Therefore, we expressed dominant negative mutant of Hrd1, Hrd1C1A, in HeLa cells stably expressing S11-NHK and S1-10 and determined its effects on NHK dislocation. Hrd1C1A is an E3-inactive form of Hrd1 that has lost its ability to ubiquitinate its substrates. Sixteen hours after transfection of Hrd1C1A, cells were treated with lactacystin for 4 h followed by immunofluorescent staining for Hrd1. Overexpression of Hrd1C1A clearly inhibited lactacystin-accumulated drGFP fluorescence, which revealed the essential role of ubiquitination for NHK dislocation in intact cells (Fig. 1C).

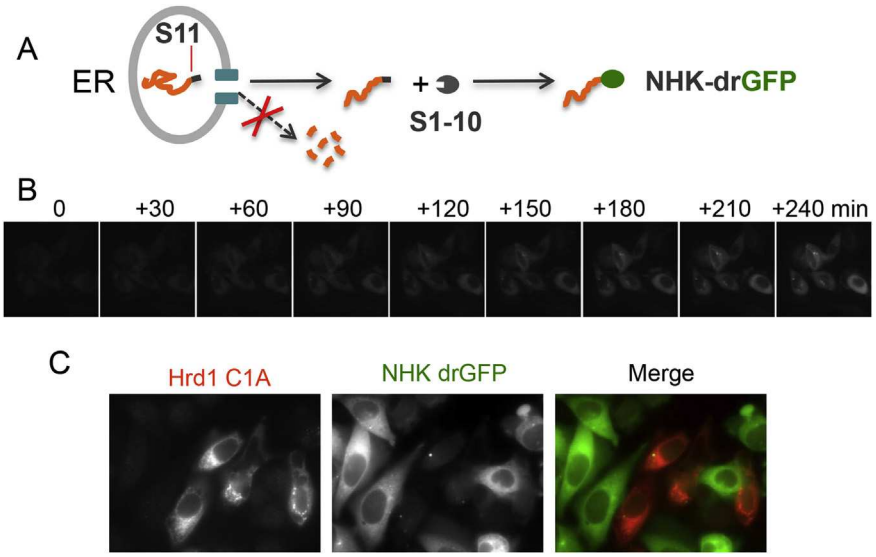

Fig. 1. Monitoring NHK dislocation by drGFP assay. A. Diagrammatic representation of drGFP assay. B. Live cell imaging of NHK dislocation in HeLa cells stably expressing S11-NHK and S1-10. C. Expression of E3-intactive mutant form of Hrd1, Hrd1C1A-myc, inhibits NHK dislocation in HeLa cells stably expressing S11-NHK and S1-10.

To take the advantage of drGFP for rapid detection of dislocation, we assessed the involvement of 21 previously isolated ERAD component proteins in NHK dislocation [12]. Of note, the function of many of the selected proteins in dislocation is unknown. To assess their involvement in NHK dislocation, the selected ERAD component proteins were knocked down in HeLa cells stably expressing S11-NHK and S1-10. Three days after siRNA transfection, cells were treated with lactacystin for 4 h to accumulate drGFP and the GFP fluorescence were measured on a TECAN fluorescence microplate reader. The results showed that most of the knockdowns either increased or had little effect on the generation of drGFP as compared to the knockdown control (Fig. 2A). Only Hrd1, Sel1L, p97/VCP, GRP94, and Derlin2 knockdown decreased the drGFP more than 40%. Surprisingly, we observed increased drGFP in cells with knockdown of importin β (Fig. 2A), since based on our previous studies [14], inhibition of importin β using a specific inhibitor importazole blocks the generation of NHK drGFP. These seemingly contradictory results could be due to the difference in the way importin β function was inhibited. Importazole treatment lasted only for 4 h, whereas knockdown lasted up to 72 h. NHK steady-state levels were expected to increase during the knockdown period, as we previously reported that knockdown of importin β blocks NHK degradation [6]. By comparison, 4-h treatment with importazole might have much less effects on NHK steady-state levels. It is reasonable to assume that the differences in the steady-state levels of NHK might affect the amount of dislocated NHK. Therefore, we detected the steady-state levels of NHK in these siRNA-transfected cells by immunoblotting (Fig. 3). As shown, NHK levels were markedly increased in cells with knockdown of importin β (Fig. 3F), suggesting that higher drGFP measurement in importin β knockdown cells may resulted from the much higher NHK levels. Similarly, knockdown of Npl4 also resulted in dramatically increased NHK levels and increased drGFP measurement (Figs. 2A and 3C). Knockdown of Derlin2 that decreased NHK drGFP, however, resulted in dramatically low NHK levels (Fig. 3E). Knockdown of SVIP decreased NHK levels while increased drGFP (Fig. 3G), which is consistent with its role as an inhibitor of ERAD [15]. Knockdown of Hrd1, Sel1L, p97/VCP or GRP94 remarkably increased NHK levels while decreased NHK drGFP, suggesting that these proteins are critically required for NHK dislocation and degradation (Fig. 3A, C, D, I).

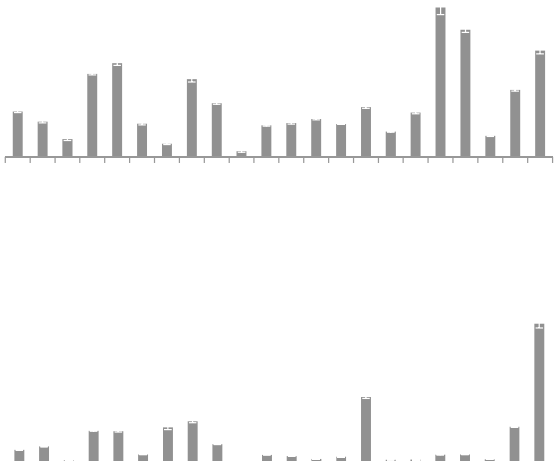

Fig. 2. RNAi screening for regulators of NHK dislocation by drGFP assay. A. Relative drGFP fluorescence intensity after knockdown of selected ERAD components. B. Relative drGFP fluorescence intensity after normalization to the steady-state levels of NHK (see Fig. 3).

To take into account the effects of NHK level differences, we normalized the drGFP measurements to the steady-state level of NHK in cells with each of the knockdowns. After normalization, we were able to classify the ERAD components examined into three groups (Fig. 2B). 1) Proteins required for NHK dislocation (normalized NHK drGFP less than 0.66). Our results suggest that Hrd1-Sel1L complex and a luminal chaperone GRP94 are critically required for NHK dislocation. Components that associated with Hrd1-Sel1L complex [22,23], including AUP1, HERP, p97/VCP-Ufd1-Npl4 complex, and Derlin1, are also required. Notably,

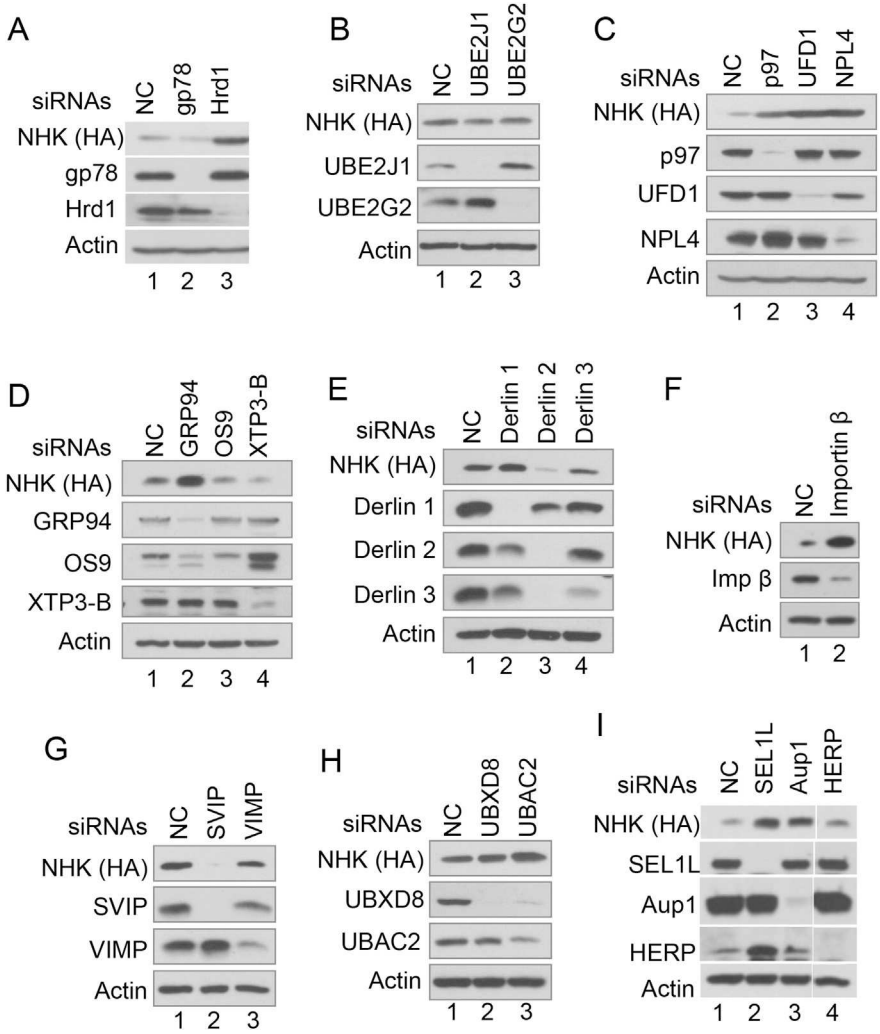

Fig. 3. RNAi of ERAD components and the resulting effects on NHK steady-state levels. Three days after siRNA transfection, cells were processed for immunoblotting as indicated.

after normalization, knockdown of importin β showed inhibition effects on NHK drGFP (Fig. 2B), suggesting its involvement in NHK dislocation as we previously reported [6,14]. In addition, two ubiquitin binding proteins UBXD8 and UBAC2 also contribute to NHK dislocation. 2) Proteins have little effect on NHK dislocation (normalized NHK drGFP less than 1.50 but more than 0.66). Interestingly, gp78 and VIMP are not required for NHK dislocation. 3) Proteins showed inhibitory effects on NHK dislocation (normalized NHK drGFP more than 1.50), including UBE2J1, UBE2G2, Derlin2, Derlin3, SVIP, OS9 and XTP3-B. Proteins in this group may inhibit NHK dislocation by preventing the formation of the ERAD complex to dislocate NHK. For an example, SVIP has been previously reported as an endogenous inhibitor of ERAD through interrupting the formation of the p97/VCP- related complexes [15]. Proteins that are less efficient or not required for NHK dislocation may also inhibit NHK dislocation by competitively binding to some key components or the substrates and preventing the formation of more efficient dislocation complexes. This may explain why Derlin1 is required for NHK dislocation whereas Derlin2 and Derlin3 inhibit that. Interestingly, both of the two E2s examined, UBE2J1 and UBE2G2, negatively regulate NHK dislocation, suggesting that some other E2s are more efficient for NHK dislocation or reduced E2 activity favors NHK dislocation.

4. Discussion

Using direct fusion of substrates with GFP as ERAD reporters, previous studies screened 59 ERAD component proteins by RNAi for their contributions to degradation of 4 different substrates [12]. They found that each substrate seems to relay on a unique set of over 10 and even 20 different ERAD components for degradation. It was suggested that ERAD system operates largely as an adaptive network, in which unique combinations of common components process individual substrates. The unique combination may form substrate-specific sub-complexes to achieve substrate-specific degradation. In this study, we extend from the previous report by screening ERAD components that contribute to dislocation, a key step in ERAD, using NHK-specific drGFP as a reporter. We screened 21 previously isolated ERAD component proteins and identified both negative and positive regulators of NHK dislocation. We also found that knockdown of one protein could affect the expression and/or stability of other ERAD components. For example, knockdown of UBE2J1 increased the levels of UBE2G2, or vice versa (Fig. 3B). Knockdown of XTP3-B increased the levels of OS9 (Fig. 3D). Knockdown of UBAC2 decreased UBXD8 (Fig. 3H). Knockdown of Sel1L increased HERP protein (Fig. 3I). These results revealed that substrate dislocation requires a complex network of many ERAD component proteins. The dislocation efficiency for

certain ERAD substrate may be determined by the levels of many proteins that negatively or positively involved. Changes in levels of any protein involved in ERAD could alter the formation of dislocation machinery, leading to enhancement or impairment of substrate dislocation.

Taking together, our studies establish substrate-specific drGFP reporter for screening for regulators of dislocation and suggest a complex scenario of dislocation in cells. Understanding the mechanisms by which these proteins regulate dislocation requires future studies.

Conflict of interest

None.

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Transparency document

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References

[1] S. Ghaemmaghami, W.K. Huh, K. Bower, R.W. Howson, A. Belle, N. Dephoure, E.K. O'Shea, J.S. Weissman, Global analysis of protein expression in yeast, Nature 425 (2003) 737–741.
[2] S.S. Vembar, J.L. Brodsky, One step at a time: endoplasmic reticulum-associated degradation, Nat. Rev. Mol. Cell. Biol. 9 (2008) 944–957.
[3] P. Ballar, Y. Shen, H. Yang, S. Fang, The role of a novel p97/valosin-containing protein-interacting motif of gp78 in endoplasmic reticulum-associated degradation, J. Biol. Chem. 281 (2006) 35359–35368.
[4] B. Tsai, Y. Ye, T.A. Rapoport, Retro-translocation of proteins from the endoplasmic reticulum into the cytosol, Nat. Rev. Mol. Cell. Biol. 3 (2002) 246–255.
[5] Z. Kostova, Y.C. Tsai, A.M. Weissman, Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation, Semin. Cell. Dev. Biol. 18 (2007) 770–779.
[6] Y. Zhong, Y. Wang, H. Yang, P. Ballar, J.G. Lee, Y. Ye, M.J. Monteiro, S. Fang, Importin beta interacts with the endoplasmic reticulum-associated degradation machinery and promotes ubiquitination and degradation of mutant alpha1-antitrypsin, J. Biol. Chem. 286 (2011) 33921–33930.
[7] J.C. Christianson, T.A. Shaler, R.E. Tyler, R.R. Kopito, OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD, Nat. Cell. Biol. 10 (2008) 272–282.
[8] D.J. van den Boomen, R.T. Timms, G.L. Grice, H.R. Stagg, K. Skodt, G. Dougan, J.A. Nathan, P.J. Lehner, TMEM129 is a Derlin-1 associated ERAD E3 ligase essential for virus-induced degradation of MHC-I, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 11425–11430.
[9] M.L. van de Weijer, M.C. Bassik, R.D. Luteijn, C.M. Voorburg, M.A. Lohuis, E. Kremmer, R.C. Hoeben, E.M. LeProust, S. Chen, H. Hoelen, M.E. Ressing, W. Patena, J.S. Weissman, M.T. McManus, E.J. Wiertz, R.J. Lebbink, A high-coverage shRNA screen identifies TMEM129 as an E3 ligase involved in ER-associated protein degradation, Nat. Commun. 5 (2014) 3832.
[10] A. Stolz, S. Besser, H. Hottmann, D.H. Wolf, Previously unknown role for the ubiquitin ligase Ubr1 in endoplasmic reticulum-associated protein degradation, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 15271–15276.
[11] E. El Khouri, G. Le Pavec, M.B. Toledano, A. Delaunay-Moisan, RNF185 is a novel E3 ligase of endoplasmic reticulum-associated degradation (ERAD) that targets cystic fibrosis transmembrane conductance regulator (CFTR), J. Biol. Chem. 288 (2013) 31177–31191.
[12] J.C. Christianson, J.A. Olzmann, T.A. Shaler, M.E. Sowa, E.J. Bennett, C.M. Richter, R.E. Tyler, E.J. Greenblatt, J.W. Harper, R.R. Kopito, Defining human ERAD networks through an integrative mapping strategy, Nat. Cell. Biol. 14 (2012) 93–105.
[13] A. Stein, A. Ruggiano, P. Carvalho, T.A. Rapoport, Key steps in ERAD of luminal ER proteins reconstituted with purified components, Cell 158 (2014) 1375–1388.
[14] Y. Zhong, S. Fang, Live cell imaging of protein dislocation from the endoplasmic reticulum, J. Biol. Chem. 287 (2012) 28057–28066.
[15] P. Ballar, Y. Zhong, M. Nagahama, M. Tagaya, Y. Shen, S. Fang, Identification of SVIP as an endogenous inhibitor of endoplasmic reticulum-associated degradation, J. Biol. Chem. 282 (2007) 33908–33914.
[16] H. Yang, X. Zhong, P. Ballar, S. Luo, Y. Shen, D.C. Rubinsztein, M.J. Monteiro, S. Fang, Ubiquitin ligase Hrd1 enhances the degradation and suppresses the toxicity of polyglutamine-expanded huntingtin, Exp. Cell. Res. 313 (2007) 538–550.
[17] B. Chen, J. Mariano, Y.C. Tsai, A.H. Chan, M. Cohen, A.M. Weissman, The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 341–346.
[18] B.N. Lilley, H.L. Ploegh, Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 14296–14301.
[19] S. Cabantous, T.C. Terwilliger, G.S. Waldo, Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein, Nat. Biotechnol. 23 (2005) 102–107.
[20] K. Nakatsukasa, G. Huyer, S. Michaelis, J.L. Brodsky, Dissecting the ER-associated degradation of a misfolded polytopic membrane protein, Cell 132 (2008) 101–112.
[21] R.M. Garza, B.K. Sato, R.Y. Hampton, In vitro analysis of Hrd1p-mediated retrotranslocation of its multispanning membrane substrate 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, J. Biol. Chem. 284 (2009) 14710–14722.
[22] E.J. Klemm, E. Spooner, H.L. Ploegh, Dual role of ancient ubiquitous protein 1 (AUP1) in lipid droplet accumulation and endoplasmic reticulum (ER) protein quality control, J. Biol. Chem. 286 (2011) 37602–37614.
[23] A. Schulze, S. Standera, E. Buerger, M. Kikkert, S. van Voorden, E. Wiertz, F. Koning, P.M. Kloetzel, M. Seeger, The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway, J. Mol. Biol. 354 (2005) 1021–1027.