The role of the ubiquitin system in human cytomegalovirus-mediated degradation of MHC class I heavy chains

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CHAPTER 5 TEB4 IS A C4HC3 RING FINGER-CONTAINING UBIQUITIN LIGASE OF THE ENDOPLASMIC RETICULUM

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Abstract

In this study, the human TEB4 is identified as a novel ER-resident ubiquitin ligase. TEB4 has homologues in many species and has a number of remarkable properties. TEB4 contains a conserved RING-finger and 13 predicted transmembrane domains. The RING finger of TEB4 and its homologues is situated at the N-terminus and has the unconventional C4HC3 configuration. The N-terminus of TEB4 is located in the cytosol. We show that the isolated TEB4 RING domain catalyses ubiquitin ligation *in vitro* in a reaction that is ubiquitin K48-specific and involves the ubiquitin-conjugating enzyme UBC7. These properties are reminiscent of E3 enzymes involved in ER-associated protein degradation (ERAD). TEB4 is an ER degradation substrate itself, promoting its own degradation in a RING finger- and proteasome-dependent fashion.

Introduction

Ubiquitination has been shown to be involved in many cellular processes, ranging from protein quality control, protein turnover, endocytosis, protein transport, signal transduction, protein translation and DNA repair (reviewed in [1]). Many of these processes are executed by regulated conditional degradation of cytosolic and ER-associated proteins. The attachment of ubiquitin to a target protein involves a cascade of enzymatic reactions, catalysed by three classes of enzymes, including the ubiquitin activating enzyme E1, ubiquitin conjugating or E2 enzymes, and ubiquitin-protein ligases or E3 enzymes [2,3]. The concerted action of these enzymes results in poly-ubiquitination of proteins. Complex formation of E3 ubiquitin ligases with E2 ubiquitin conjugating enzymes provides substrate specificity to the ubiquitination reaction. The fate of a ubiquitinated molecule is not only influenced by the E2-E3 complex involved in the ubiquitination reaction, but also by the site of ubiquitination, the number of ubiquitin moieties attached, and the lysine residues of ubiquitin involved in the linkage.

In humans, three classes of E3 ligases can be distinguished. The first is a group of proteins carrying a 350-residue C-terminal HECT domain. Examples are the human papilloma virus E6-Associated Protein, a co-factor in the degradation of the tumour suppressor gene p53 [4], and Smurf, which has a role in embryonic pattern formation [5]. The second class is formed by U-box containing E3's like the Carboxyl terminus of Hsc70-Interacting Protein (CHIP) [6]. The third class harbours RING-finger containing E3 ligases like c-Cbl, which down-regulates receptor tyrosine kinases [7], and a diverse group of

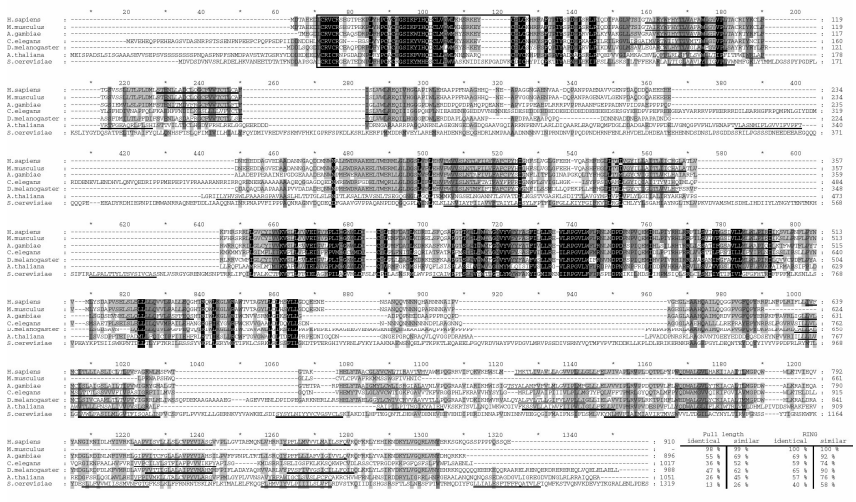

Figure 1. Amino acid sequence alignment of TEB4 homologues.
TEB4 of Homo sapiens (T00268), Mus musculus (NP_766194), Anopheles gambiae (EAA04193), Caenorhabditis elegans (NP_492823), Drosophila melanogaster (NP_647715), and Arabidopsis thaliana (NP_195136) are putative proteins in the database, whereas Saccharomyces cerevisiae Doa10 (P40318) has been cloned [31]. The RING finger is boxed and the predicted transmembrane domains are underlined. Hydropathy analysis of TEB4 was performed using www.ch.embnet.org/software/TMPRED _form.html and http://cubic.bioc.columbia.edu/predictprotein. The percentage of amino acid identity and similarity to HsTEB4 are indicated for the full-length sequences and the RING domains of the TEB4 homologues.

membrane proteins including HRD1, GP78/AMFR, and the viral K3-related MARCH proteins [8-17].

A RING finger consists of a double ring structure containing 8 metal binding cysteine and histidine residues that coordinate two zinc ions. RING fingers of E3 ligases can be formed by different configurations of histidine and cysteine residues. The most frequently found 'classical' C3HC4 RING domains are involved in many different cellular events. Examples are c-CBL, which functions in ubiquitin-dependent lysosomal trafficking [7,18,19], and BRCA1, which affects cell cycle progression through its ligase activity via a mechanism that is still elusive [20,21]. RING fingers with a C3H2C3 configuration are found in membrane-associated E3 ligases catalysing ubiquitination of degradation substrates occurring in the secretory pathway, especially the ER, and endolysosomal compartments. So far, three ER-resident membrane E3 ligases with C3H2C3 RING fingers have been identified: yeast HRD1/Der3 [8,9], its human homologue HRD1 [15-17], and the mammalian gp78/AMFR [10]. Some proteins carry atypical forms of RING domains, such as the 'double RING finger' found in Parkin, an E3 ligase

implicated in Parkinson's disease. Parkin is one of the proteins that suppress unfolded protein-induced cell death through their E3 ubiquitin-protein ligase activity [22]. A third, frequently occurring RING finger configuration is C4HC3. Several C4HC3 RING finger proteins, including the yeast Doa10, the murine gammaherpesvirus 68 mK3, the Kaposi sarcoma herpesvirus kK3 and kK5, the myxomavirus M153R, and the human MARCH proteins, have been found to possess E3 ubiquitin ligase activity [11-14,23-30].

In this study, we identify the C4HC3 RING finger-containing TEB4, originally characterized as a transcript of the Cri-du-chat critical region on chromosome 5, as a novel ER-associated E3 ligase. TEB4 appears to be well conserved, as genes with a high degree of homology to TEB4 occur in many species. *In vitro* ubiquitination reactions indicate that the N-terminal RING finger of TEB4 acts in conjunction with the E2 enzyme UBC7 and specifically catalyzes conjugation of ubiquitin through its lysine at position 48.

Results

Structural features of TEB4

An alignment of human TEB4 with related protein sequences in the mouse, C. elegans, Anopheles, Arabidopsis, and Drosophila indicates that the TEB4 sequences are well conserved (Figure 1). The overall homology of TEB4 to the Ssm4 protein or Doa10, the proposed yeast homologue of TEB4, predominantly occurs in the N-terminal RING domains and a region ranging from residue 650-800, designated the TEB4-Doa10 domain [23]. A conserved RING finger is predicted at the very N-terminus of human, mouse, Anopheles, and Drosophila TEB4 (boxed in Figure 1). The homologues in C. elegans, Arabidopsis, and yeast have an additional sequence that precedes the RING domain.

In vivo and *in vitro* expression of TEB4

TEB4 is expressed in many different organs, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, and small intestine [31], suggesting that it is an essential protein, involved in general housekeeping functions in the cell. Since TEB4 is encoded in a chromosome region associated with a neurodegenerative disorder, the Cri-du-chat syndrome, expression of the protein was investigated in a homogenate of human brain tissue. Detection of TEB4 by Western blotting using a TEB4-specific antiserum revealed a major polypeptide band of approximately 97 kD (Figure 2A, lane 1). The antiserum did not cross-react with yeast Doa10 (Figure 2A, lane 2) but did recognize the TEB4 fusion protein that was used to generate the antiserum (Figure 2A, lane 3).

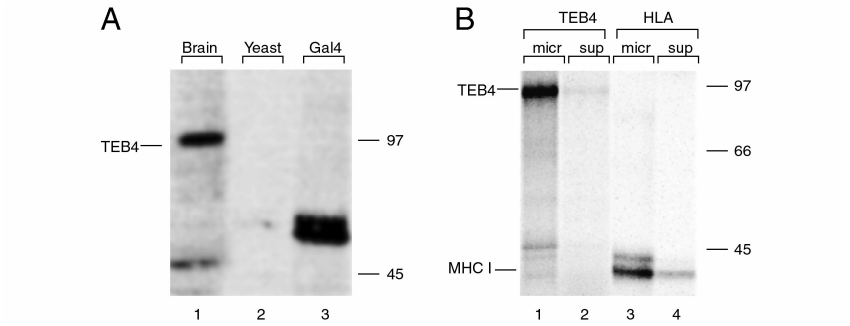

Figure 2. Expression of TEB4 *in vivo* and *in vitro*.
(A) TEB4 expression in human brain was detected using a rabbit serum directed against amino acids 482-971 of KIAA0597 (Lane 1). Yeast homogenate was used as a negative control (Lane 2). The fusion protein TEB4(482-971)-GAL4 DNA-binding domain (used to generate the antibodies) served as a positive control (Lane 3). Molecular weights are in kiloDaltons. (B) TEB4 and HLA-A2 were translated *in vitro* in the presence of dog pancreas microsomes and L-[35S]-methionine. 1/10 of the microsomal fractions (micr.) and 1/2 of the supernatant fractions (sup.) were loaded on SDS-acrylamide gel.

In vitro transcription and translation of TEB4 cDNA in the presence of microsomal membranes also yielded a translation product of 97 kD (Figure 2B, lane 1). The polypeptide predominantly occurred in the microsomal fraction, which suggests that TEB4 is inserted into the membrane (Figure 2B, compare lanes 1 and 2). Likewise, the *in vitro* translated MHC class I heavy chain HLA-A2, a type I membrane protein with a single transmembrane domain, was mostly detected in the membrane fraction (lanes 3 and 4).

Membrane topology of TEB4

The hydropathy analysis of TEB4 suggests 13 transmembrane regions (underlined in Figure 1). The number and distribution of hydrophobic sequences varies somewhat among the TEB4 homologues. The putative membrane topology of human TEB4 is presented in figure 3A. In view of the proposed E3 ligase activity of the RING finger, this domain has been positioned in the cytosol. To confirm the cytosolic disposition of the RING domain we used two different approaches.

First, we performed proteinase K digestions on canine pancreatic microsomes containing in vitro translated TEB4. If the model shown in Figure 3A is correct, proteinase K digestion of the microsomes should result in the loss of an N-terminal epitope, whereas a C-terminal epitope should be protected. Treatment of the microsomes with proteinase K caused a dose-dependent loss of translation product (Figure 3B, left panel). Whereas an antibody directed against the N-terminal region of TEB4 recovered the intact protein from untreated samples, proteinase K digestion resulted in a loss of the epitope (Figure 3B, middle panel). An antibody against the C-terminal

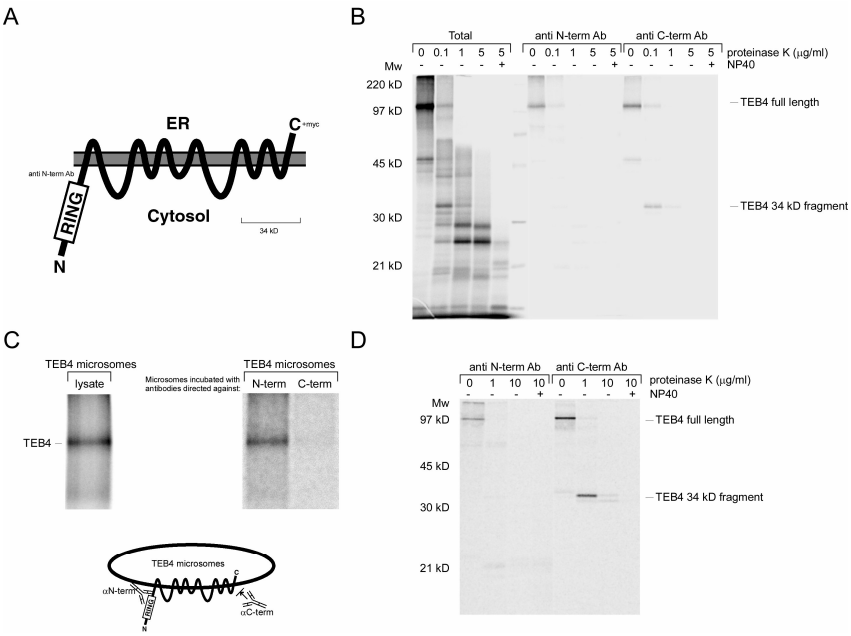

Figure 3. Membrane topology of TEB4.
(A) Predicted membrane topology of TEB4, based on analyses with software available on www.ch.embnet.org/software/TMPRED and www.cubic.bioc.columbia.edu/predictprotein. (B) TEB4 was translated *in vitro* in the presence of dog pancreas microsomes. After translation, the microsomes were subjected to proteinase K digestion in KMH buffer or Np40 as indicated. 1/3 of the microsomes were loaded directly on gel (Total, left panel) and the other 2/3 were lysed and subjected to immunoprecipitation with either an antibody directed against the N-terminus of TEB4, or anti-myc antibody directed against the myc tag fused to the C-terminus of TEB4. (C) TEB4 was translated *in vitro* in the presence of microsomes. The total amount present in the microsomes is shown in the left panel. Intact microsomes were incubated with the same antibodies as in B for 2 hours as indicated in the cartoon. The microsomes were washed to remove excess of antibodies and lysed. The cleared supernatant was incubated with Protein A-Sepharose beads to isolate TEB4-antibody complexes. (D) TEB4 was expressed in TS20 cells and subjected to proteinase K digestion as described in B. After digestion, lysates were prepared and immunoprecipitations were performed with the antibodies indicated.

myc-tag was able to precipitate a 34 kD TEB4 fragment from proteinase K-treated microsomes, suggesting a luminal deposition of the C-terminus. At higher proteinase K concentrations the 34 kD fragment disappeared, presumably due to proteolysis by the enzyme.

Secondly, microsomes containing in vitro translated TEB4 were incubated with antibodies directed against either the N- or C-terminus of TEB4. Subsequently, the microsomes were washed to remove excess of antibody and lysed. The lysates were incubated with Protein A-Sepharose beads to isolate the antibodies that had bound to microsome-associated TEB4. Antibodies directed against the N-terminal epitope were capable of

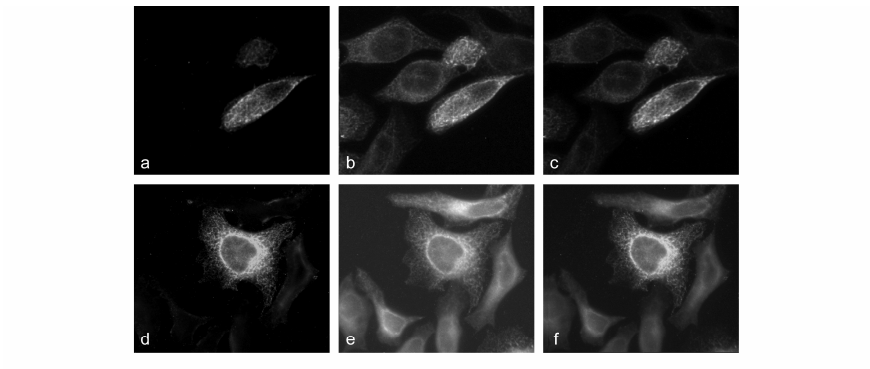

Figure 4. Subcellular localization of TEB4.
TEB4 (panels a, b and c) and TEB4C9A RING mutant (panels d, e and f), both tagged with a myc epitope at their C-terminus, were transiently expressed in HeLa cells. Myc-TEB4 protein and endogenously expressed calnexin were detected by immunofluorescence using antibodies against myc-tag (panels a, d; red) and anti-calnexin (panels b, e; green), respectively. An overlay of TEB4 and calnexin staining is shown in panels c and f.

precipitating TEB4, whereas the antibody directed against the C-terminal epitope failed to precipitate TEB4, indicating that the N-terminus, but not the C-terminus of microsome-inserted TEB4 was accessible to the antibodies (Figure 3C).

To confirm the results obtained with in vitro translated TEB4, the proteinase K digestion experiments were also performed on cells expressing TEB4. Figure 3D shows that the antibody directed against the N-terminus of TEB4 failed to bind TEB4 when the cells were permeabilised and treated with proteinase K. The antibody directed against the C-terminus of TEB4 immunoprecipitated the 34 kD fragment that was also observed in proteinase K-treated microsomes. From the combined experiments we conclude that the N-terminal region of TEB4 is located in the cytoplasm, whereas the C-terminus of TEB4 has a luminal deposition.

Subcellular localization of TEB4

To investigate the intracellular localization of TEB4, Myc-tagged wild type TEB4 and a TEB4 RING finger mutant were expressed in HeLa cells and stained using immunofluorescence. TEB4 staining patterns are reminiscent of ER localization (Figure 4A and D), which was confirmed by co-localization of TEB4 with the ER-resident chaperone calnexin (Figure 4C and F). No TEB4 was detected when non-permeabilised cells were used, indicating that TEB4 is not expressed at the cell surface (data not shown). ER localization is a conserved characteristic of human TEB4, yeast Doa10, and some of the human MARCH proteins [13,23].

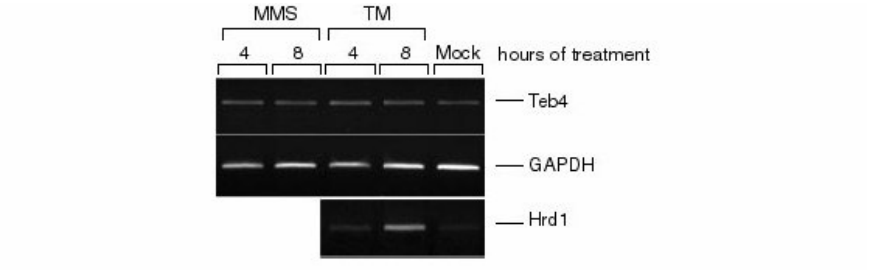

Figure 5. TEB4 expression upon stress induction.
HeLa cells were treated with 1mM methyl methanesulfonate (MMS) or 10 μg/ml tunicamycine for 4 or 8 hours. Total RNA was prepared and subjected to semi-quantitative RT-PCR using primers specific for TEB4 and HRD1.

TEB4 expression is not induced by ER stress

The accumulation of unfolded or malfolded proteins in the lumen of the ER leads to a stress response, the 'unfolded protein response' (UPR). Unfolded proteins can be removed by folding or degradation. During UPR, the synthesis of ER-resident protein chaperones is increased, which enhances the folding capacity in this organelle. At the same time, proteins engaged in ER-associated protein degradation, such as HRD1/Der3, are upregulated. [15,17,32-34].

To establish whether TEB4 is up-regulated upon induction of UPR, we examined the expression of TEB4 mRNA under conditions of cellular stress. Cells were treated with the genotoxic stress inducer methyl methanesulfonate (MMS), or with the N-glycosylation inhibitor tunicamycin [35]. Due to inhibition of N-linked glycosylation, tunicamycin interferes with proper folding of many glycoproteins, thereby inducing the ER-specific UPR. TEB4 mRNA levels were determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using specific primers. The results shown in Figure 5 indicate that TEB4 expression does not increase in response to the various stress conditions. The mRNA level of HRD1/Der3, a known ER stress response gene product, was increased in the presence of tunicamycin, indicating induction of ER stress in the cells.

TEB4 is a RING finger-dependent ER degradation substrate

A specific characteristic of several E3 ligases analysed is the capacity to catalyse their own degradation in a RING finger-dependent fashion. We therefore evaluated the stability of TEB4 and its RING finger mutant. HeLa cells were transiently transfected with Myc-tagged wild-type and RING-mutant TEB4 and a chase was performed in the presence of cycloheximide. The results shown in Figure 6A indicate that wild type TEB4 is completely

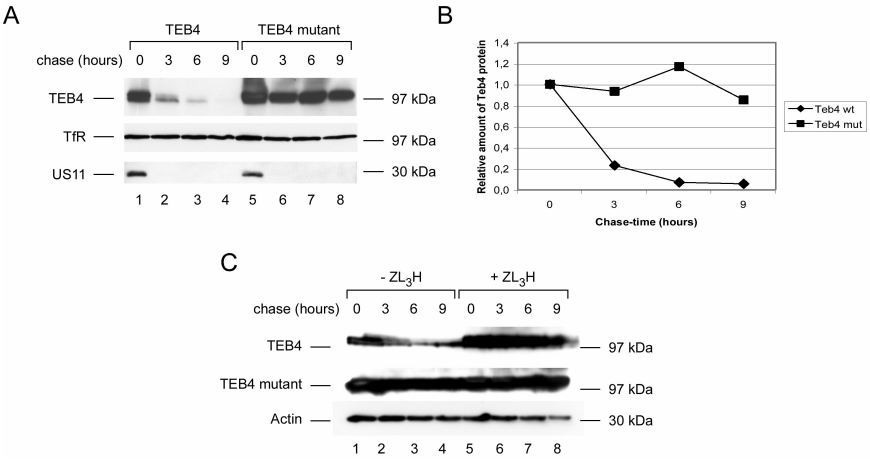

Figure 6. TEB4 is a RING finger-dependent ER degradation substrate.
(A) HeLa cells stably transfected with HCMV-US11 were transiently transfected with myc-tagged TEB4 or TEB4C9A RING mutant. 24 hours after transfection, the cells were treated with 50 μg/ml cycloheximide and harvested at the chase times indicated. Cell lysates were subjected to SDS-PAGE and stained by Western blotting using antisera against myc-tag, US11 or human transferrin receptor (TfR). (B) Quantification of the Western blots shown in A. (C) Same experiment as in A, in the presence or absence of the proteasome inhibitor ZL_3H.

degraded within 9 hours of chase (lanes 1-4). The C9A mutation within the RING domain of TEB4 strongly impaired its degradation (lanes 5-8). Transferrin receptor remained stable throughout the chase and served as a loading control. The HCMV-encoded US11 was degraded in the presence of both wild type and RING finger mutant TEB4, indicating that degradation of an ER protein proceeds as normal in TEB4 mutant-transfected cells. A quantification of TEB4 levels detected in the Western blots is shown in Figure 6B.

To investigate whether the degradation of TEB4 involves proteasomes, the pulse-chase experiment was performed in the presence of the proteasome inhibitor ZL_3H. As shown in Figure 6C (upper panel), inhibition of proteasomes results in stabilization of TEB4 up to 9 hours after addition of cycloheximide. These results indicate that TEB4 catalyzes its own degradation in a RING finger- and proteasome-dependent fashion.

The TEB4 RING domain interacts with UBC7 and promotes K48-specific ubiquitin to ubiquitin linkage.

In the yeast *Saccharomyces cerevisiae*, the ER-tethered E3 ligases Hrd1 and Doa10 interact specifically with the ubiquitin-conjugating enzyme UBC7 [8,9,23]. We have previously shown that the RING domain sequence in the human and yeast ER-tethered E3 ubiquitin ligase Hrd1p interacts with the ubiquitin-

conjugating enzyme UBC7 and generates a robust activity for the K48-specific linkage of ubiquitin to ubiquitin [17]. We produced the TEB4 RING domain as a GST-fusion protein in bacteria and tested the purified the protein for its interaction with UBC7 with a similar *in vitro* ubiquitination assay. Similar to what we observed with the GST-Hrd1 RING fusion, the inclusion of GST-TEB4 RING domain in an *in vitro* UBC7-dependent ubiquitination reaction also produced a reaction product that migrated in SDS-gels with a mobility expected of a ubiquitin dimer (Figure 7A, lane 4). This activity was absent when UBC7 was replaced either by UBC5, UBC2 or UBC3 (data not shown). The formation of the reaction product is dependent on the presence of K48 in ubiquitin (Figure 7A, lane 5), consistent with it being a K48-linked ubiquitin dimer. To demonstrate directly the formation of a K48-linked ubiquitin dimer, we analysed the tryptic digests of the reaction product by mass spectrometry. For comparison, we carried out a similar analysis for ubiquitin. In a K48-linked ubiquitin dimer, K48 that is linked to the C-terminal carboxyl of another ubiquitin becomes resistant to tryptic digestion [36] and retains minimally, via an isopeptide linkage, the two C-terminal glycine residues of the other ubiquitin, when all other sites are cleaved. This isopeptide has a unique mass of 1460.75 that is not found in ubiquitin. In our analysis, we detected a peptide with a mass of 1460.78, in addition to all the peptides that are also found in the single ubiquitin sample. Thus, we conclude that the TEB4 RING sequence interacts with UBC7 and promotes the linkage of ubiquitin to ubiquitin at K48.

The UBC7-dependent ubiquitin-to-ubiquitin linkage reaction requires the presence of the ubiquitin-activating enzyme E1 and ATP, suggesting that the activity requires the intermediate formation of a UBC7-ubiquitin thioester. We next addressed whether the activity required one or both ubiquitin molecules to be linked by thioester to UBC7. To test these two possibilities, we generated two distinct forms of ubiquitin, one that can form a thioester linkage with UBC7 but lacks K48 (UbK48R) and another ubiquitin that lacks the C-terminal Gly-Gly residues (Ub_{74}) and is unable to be activated by the ubiquitin-activating enzyme. To distinguish these two forms of ubiquitin, we extended the N-terminus of Ub-K48R with a MCHHHHHH sequence and attached a fluorescent Oregon Green label at the cysteine residue, using a previously described procedure [37]. Figure 7C shows that the UBC7-linked Oregon Green-labelled Ub-K48R can be transferred to Ub_{74} in a reaction that is dependent on the presence of either GST-HRD1 RING or GST-TEB4 RING. The reaction requires the presence of K48 in Ub_{74} since a similar ubiquitin dimer was not formed with the K48R mutant of this truncated ubiquitin (data not shown). These results suggest that the ubiquitin dimer is formed by a reaction as depicted in Figure 7B and is reminiscent of the

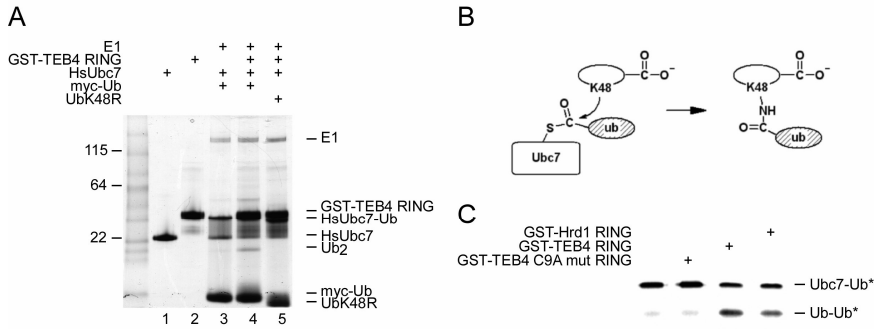

Figure 7. TEB4 facilitates a K48R-specific ubiquitination ligation reaction.
(A) The TEB4 RING finger facilitates the *in vitro* K48-specific linkage of ubiquitin to ubiquitin. Protein components as indicated were incubated at 30 °C for 10 minutes in a reaction buffer containing 25 mM Tris (pH7.6), 10 mM $MgCl_2$ and 1 mM ATP. When indicated, the reaction contains 10 nM E1, 10 μM UBC7, 10 μM GST-TEB4 RING, and 50 mM polyHis-tagged ubiquitin or the ubiquitin mutant Ub-K48R. Reactions were stopped by the addition of non-reducing SDS-sample buffer, and protein components were then separated by SDS-PAGE and visualized by staining with Coommassi brilliant blue. The migration of purified HsUbc7 and GST-TEB4 RING are shown in lanes 1 and 2, respectively. A ubiquitin dimer is formed in the reaction containing E1, GST-TEB4 RING, UBC7 and ubiquitin (lane 4) but not when ubiquitin was replaced by the mutant Ub-K48R (lane 5) or in the absence of GST-TEB4 RING (lane 3). (B) Schematic representation of the transfer of thioester-linked ubiquitin on UBC7 to a free ubiquitin. (C) A Ubiquitin dimer is formed by the transfer of thioester-linked ubiquitin on UBC7 to a free ubiquitin. A reaction mixture containing 25 mM Tris (pH7.6), 1 mM $MgCl_2$, 0.5 mM ATP, 1 nM E1, 1 μM UBC7 and 2 μM Oregon Green-labeled ubiquitin [37] was incubated at 30 °C for 1 minute to generate UBC7-ubiquitin thioester (UBC7-ub). The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. The mixture was then incubated at 30 °C for 15 minutes with the addition of 40 μM of a truncated ubiquitin that lacks the C-terminally Gly-Gly residues and 10 μM of RING proteins as indicated. At the end of the incubation, the reaction was stopped by the addition of non-reducing SDS-sample buffer. Proteins were separated by SDS-PAGE and proteins bearing the Oregon-green fluorescence (UBC7-ub thioester and ubiquitin dimer) were visualized on a fluorescence gel scanner. Free ubiquitin migrated at the dye front and its fluorescence is masked by the presence of bromophenol blue in the SDS-sample buffer and is not shown here.

ubiquitin-to-ubiquitin linkage reaction during polyubiquitin chain elongation.

Discussion

In this study, TEB4 was characterized as a C4HC3 RING finger-containing E3 ubiquitin ligase of the endoplasmic reticulum. RING domains of E3 enzymes serve as a binding motif for ubiquitin conjugating enzymes (E2's). The N-terminal C4HC3 RING finger of TEB4 is likely to fulfil a similar function, as we have found the TEB4 RING domain to catalyse lysine 48-dependend ubiquitin ligation in the presence of the E2 UBC7.

C4HC3 RING fingers have been found in several viral, yeast, and mammalian E3 ligases. Viral C4HC3 RING finger proteins are the murine gammaherpesvirus 68 mK3, the Kaposi sarcoma herpesvirus kK3 and kK5,

and the myxomavirus M153R proteins [11,24-27,29,30,38-40]. Interestingly, these viral E3 ligases all inhibit the expression of mammalian proteins playing a role in anti-viral immunity, i.e. MHC class I complexes and the co-stimulatory molecules ICAM-1 and B7.2 [24-27,29,30,38-40]. Doa (*d*egradation *o*f *d*lpha2)-10 [23], also designated Ssm4 [41], was identified as a ubiquitin ligase that promotes the degradation of the soluble transcription factor Matα2, in addition to degradation of proteins carrying a Deg1 signal (an exposed hydrophobic face of an amphipathic helix) [42]. Moreover, Doa10 and Hrd1p together are able to suppress growth defects due to malfunctioning of the Cdc48p-Npl4p-Ufd1p complex related to a temperature-sensitive mutation in Npl4p [43]. Recently, Doa10 and Hrd1p were shown to be involved in the ubiquitin-dependent degradation of human CFTR ectopically expressed in yeast [44].

Human C4HC3 RING-containing proteins for which E3 ligase activity has been demonstrated are MEKK1 [45] and a family of proteins called Membrane Associated RING-CH (MARCH) proteins [13]. MEKK1 is a cytosolic protein catalysing ubiquitination of Extracellular signal-Regulated protein Kinases (ERK)-1 and 2 [45], members of the MAP kinase family. Apart from a similar RING motif, MEKK1 does not demonstrate any obvious similarity to the MARCH proteins or TEB4 with respect to structure or subcellular localization. Interestingly, the MARCH proteins share several structural and functional properties with the viral K3 proteins [13]. Substrate specificity of the MARCH proteins and the viral E3 ligases demonstrates considerable overlap, with MHC class I molecules being one of the common substrates.

Ubiquitin can form isopeptide linkages involving its lysines at positions 6, 11, 29, 48 and 63. The different ubiquitin linkages are associated with various functions of ubiquitin within the cell. K63-based ubiquitin linkages have been associated with DNA repair, IκB kinase activation and endocytoses (reviewed in [1,46]). The use of ubiquitin residue K48 has been linked to proteasomal degradation and dislocation of proteins from the ER into the cytosol [37,47] and binding to the AAA ATPase p97-Ufd-Npl4 complex [48,49]. K11 and K29 have been associated with protein degradation by the proteasome as well.

The observation that the isolated RING domain of TEB4 catalyses an *in vitro* ubiquitination reaction involving lysine 48 of ubiquitin points towards a function in proteasomal degradation. Moreover, TEB4 catalyses its own degradation in a RING finger- and proteasome-dependent fashion and acts in conjunction with UBC7. These observations, combined with the association of many other C4HC3 RING-containing E3 ligases with protein degradation, led us to investigate whether TEB4 might have a similar function. Surface

expression of MHC class I, Fas, TfR, CD4, and B7.2 molecules, however, was not influenced by overexpression of TEB4 or its RING finger mutant (data not shown). Previously, others and we have shown that dislocation and degradation of MHC class I heavy chains in the context of human cytomegalovirus US11 is ubiquitin-dependent [37,50]. The E2 and E3 enzymes involved in this process remain to be identified. Expression of neither wild type nor RING finger mutant TEB4 had any effect on US11-mediated degradation of class I heavy chains (data not shown).

The observation that TEB4 degradation involves the RING domain of TEB4 itself suggests that TEB4 catalyses its own degradation. This self-induced degradation may play a role in the regulation of TEB4 expression, as has been suggested for TRAF2 [51].

An interesting property shared by HRD1, gp78, Doa10, and TEB4 is their large number of putative transmembrane domains. HRD1, gp78, TEB4, and Doa10p are predicted to contain 6, 7, 13, and 13 membrane-spanning sequences, respectively. In view of the function of HRD1 and gp78 in ERAD [10,17], one could speculate that their transmembrane domains might form part of a channel that mediates retrograde movement of substrates from the ER into the cytosol. These E3 ligases might associate with Sec61 alpha and beta translocon subunits that are also believed to be involved in dislocation of ER proteins [52-54]. These E3 ligases may also act in conjunction with Derlin-1, a protein that has recently been found to be required for US11- (but not US2-) dependent dislocation of MHC class I heavy chains [55,56]. Further studies will be required to investigate whether multi-membrane spanning E3 ligases indeed form part of a 'dislocon'.

Materials & Methods

Plasmids

The KIAA0597 cDNA, encoding the complete sequence of TEB4, was obtained from the HUGE sequencing project [31]. A PCR fragment with the KIAA0597 sequence beginning at the first ATG codon of the ORF, flanked by an EcoRI site at the 5' end and a KpnI site at the 3' end was cloned into pcDNA3.1 myc/His A (-) vector (Invitrogen) to obtain a plasmid containing C-terminal myc- and His-tags. A mutation of the first cysteine of the RING finger into an alanine was accomplished using the Quick Change Site Directed Mutagenesis kit (Stratagene), resulting in a product designated TEB4C9A. The plasmid containing the HLA-A0102 sequence has been described [50].

Antibodies

Anti-myc antibodies were from Roche (used for immunoblotting and immunoprecipitations) or Invitrogen (used for immunoprecipitations and immunofluorescence analysis). Anti-US11 antibody was produced in rabbits as described [50]. Anti-calnexin antiserum was a kind gift from Dr. A. Helenius, Institute of Biochemistry, ETH Zurich, Switzerland. The anti-transferrin receptor monoclonal antibody was from Roche. Anti TEB4 antiserum was produced in rabbits using a fusion protein of TEB4 (amino acids 482-971) and the GAL4 DNA binding domain. The antibody directed against the N-terminus of TEB4 was produced as described [50] using a peptide derived from amino acids 65-87 of TEB4. Horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories.

Immunofluorescence analysis

HeLa cells were transfected with myc-tagged TEB4 or TEB4C9A-encoding plasmids using Lipofectamine Plus™ (Invitrogen). After 24 hours, cells were fixed with 3% paraformaldehyde in PBS for 10 minutes and permeabilized for 5 minutes using 0.2% Triton X-100 in PBS. Cells were stained with anti-Myc mAb or anti-calnexin rabbit serum, followed by CY3-conjugated anti-mouse and FITC-conjugated anti-rabbit serum.

In vitro transcription and translation, and proteinase K digestions

TEB4 and HLA-A0201 plasmids were linearized with AflII and XhoI, respectively, and used for *in vitro* transcription with T7 polymerase (Promega). Transcripts were translated in the presence of L-[35S]methionine in rabbit reticulocyte lysate containing canine pancreatic microsomes. Reactions of 25 µl were incubated at 30°C for 90 minutes. Microsomes and supernatant were separated by centrifugation at 14,000g at 4°C for 15 minutes. The pellets were washed twice with KMH buffer (110 mM KAc, 2 mM MgAc and 20 HEPES-KOH pH 7.2) and 1mM $CaCl_2$ prior to loading onto SDS polyacrylamide gel. Proteins were displayed using a Personal Molecular Imager FX phosphor imager (BioRad).

For proteinase K digestions, microsomal membranes were centrifuged at 14,000 *g* for 15 minutes and washed with 100 µl KMH buffer containing 110 mM KAc, 2 mM MgAc and 20 HEPES-KOH pH 7.2. Proteinase K digestions were performed in 50 µl KMH or NP40 lysis mix on ice for 30 minutes. Proteinase K was used at the concentrations indicated. After digestion, 1 µl 500 mM PMSF was added to the NP40 samples, whereas 200 µl KMH containing 4 mM PMSF was added to the KMH samples. The microsomes were centrifuged at 14,000 *g* for 15 minutes and resuspended in

60 µl NP40 lysis mix containing 1 µl 500 mM PMSF. After lysis for 20 minutes, samples were cleared by centrifugation at 14,000 *g* for 15 minutes. The supernatant was split and used for either direct loads or immunoprecipitations.

For proteinase K digestions on whole cells, cells were infected with recombinant vaccinia expressing T7 [57] and after one hour transfected with pcDNA3.1 TEB4 myc/his plasmid containing the T7 promotor in front of the TEB4 ORF. After another three hours, cells where pulsed for one hour, labelled with ^{35}S-Redivue Promix, and subsequently resuspended in 100 µl of cold permeabilization buffer (containing 25 mM Hepes, pH 7.2, 115 mM potassium acetate, 5 mM sodiumacetate, 2.5 mM $MgCl_2$, 0.5 mM EGTA, and 400 µg/ml digitonin (Calbiochem)) or NP40 lysis mix. Proteinase K (LifeTechnologies) was added at the concentrations indicated. Permeabilized cells were incubated with the digestion mix at 4 °C for 20 min. Proteolysis was stopped by centrifugation of the cells at 14,000 *g* for 10 min and resuspension of the pellets in 1 ml of NP-40 lysis buffer containing 1 mM PMSF. Immunoprecipitations, SDS-PAGE, and PhosphorImaging were performed as described [50].

In Vitro Ubiquitination Assays

Bacterially expressed glutathione S-transferase (GST)-TEB4-RING or GST-TEB4 RING-mutant fusion proteins were obtained using PCR fragments encoding residues 1–69 of human TEB4 or TEB4-C9A. EcoRI and XhoI sites were included in the PCR primers. The resulting PCR fragment was inserted into the pGEX-5X-3 vector (Amersham). *In vitro* ubiquitination assays were carried out at 30 °C in a reaction mixture containing 25 mM Tris (pH7.6), 10 mM $MgCl_2$, and 1 mM ATP [17]. When indicated, the reaction contained E1, human UBC7, GST-TEB4 RING, and ubiquitin, the ubiquitin mutant UbK48R, or modified ubiquitin. Reactions were terminated by the addition of SDS sample buffer, and protein components were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. For fluorescent *in vitro* ubiquitination experiments, his-tagged K48R-ubiquitin was modified with the fluorescent probe Oregon Green. K48R-ubiquitin was labelled chemically by fusing 2-,7-difluorofluorescein (Oregon Green) iodoacetamide to the cysteine residue and purified by size exclusion chromatography as described earlier [37].

Peptide Mass Analysis

Ubiquitin or ubiquitin dimer were excised from SDS-gels and digested with trypsin using an in-gel digestion protocol [58]. MALDI-TOF (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry)

analysis of tryptic peptide mass analysis was performed by the fee-for-service Penn State Core Facility.

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References

1 Fang, S. and Weissmann, A. (2004) Ubiquitin-proteasome systemA field guide to ubiquitylation. Cell Mol.Life Sci. **61**, 1546-1561

2 Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. Annu.Rev.Biochem. **67**, 425-479

3 Pickart, C. (2001) Mechanisms underlying ubiquitination. Annu.Rev.Biochem. **70**, 503-533

4 Beer-Romero, P., Glass, S. and Rolfe, M. (1997) Antisense targeting of E6AP elevates p53 in HPV-infected cells but not in normal cells. Oncogene **14**, 595-602

5 Zhu, H., Kavsak, P., Abdollah, S., Wrana, J. and Thomsen, G. (1999) A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature **400**, 687-693

6 Jiang, J., Ballinger, C., Wu, Y., Dai, Q., Cyr, D., Hohfeld, J. and Patterson, C. (2001) CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J.Biol.Chem. **276**, 42938-42944

7 Joazeiro, C., Wing, S., Huang, H., Leverson, J., Hunter, T. and Liu, Y. (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science **286**, 309-312

8 Bays, N., Gardner, R., Seelig, L., Joazeiro, C. and Hampton, R. (2001) Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat.Cell Biol. **3**, 24-29

9 Bordallo, J., Plemper, R., Finger, A. and Wolf, D. (1998) Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol.Biol.Cell **9**, 209-222

10 Fang, S., Ferrone, M., Yang, C., Jensen, J., Tiwari, S. and Weissman, A. (2001) The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. Proc.Natl.Acad.Sci.U.S.A **98**, 14422-14427

11 Coscoy, L. and Ganem, D. (2003) PHD domains and E3 ubiquitin ligases: viruses make the connection. Trends Cell Biol. **13**, 7-12

12 Fruh, K., Bartee, E., Gouveia, K. and Mansouri, M. (2002) Immune evasion by a novel family of viral PHD/LAP-finger proteins of gamma-2 herpesviruses and poxviruses. Virus Res. **88**, 55-69

13 Bartee, E., Mansouri, M., Hovey Nerenberg, B., Gouveia, K. and Fruh, K. (2004) Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. J.Virol. **78**, 1109-1120

14 Aravind, L., Iyer, L. and Koonin, E. (2003) Scores of RINGS but No PHDs in Ubiquitin Signaling. Cell Cycle **2**, 123-126

15 Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M. and Nomura, Y. (2002) Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. FEBS Lett. **532**, 147-152

16 Nadav, E., Shmueli, A., Barr, H., Gonen, H., Ciechanover, A. and Reiss, Y. (2003) A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. Biochem.Biophys.Res.Commun. **303**, 91-97

17 Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., Van Voorden, S., Thanedar, S., Roitelman, J., Chau, V. and Wiertz, E. (2003) Human Hrd1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J.Biol.Chem. **279**, 3525-3534

18 Levkowitz, G., Waterman, H., Ettenberg, S., Katz, M., Tsygankov, A., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S. and Yarden, Y. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol.Cell **4**, 1029-1040

19 Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W., Zhang, H., Yoshimura, A. and Baron, R. (1999) Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. J.Biol.Chem. **274**, 31707-31712

20 Ruffner, H., Joazeiro, C., Hemmati, D., Hunter, T. and Verma, I. (2001) Cancer-predisposing mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity. Proc.Natl.Acad.Sci.U.S.A **98**, 5134-5139

21 Choudhury, A., Xu, H. and Baer, R. (2004) Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression. J.Biol.Chem. **279**, 33909-33918

22 Imai, Y., Soda, M. and Takahashi, R. (2000) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J.Biol.Chem. **275**, 35661-35664

23 Swanson, R., Locher, M. and Hochstrasser, M. (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev. **15**, 2660-2674

24 Boname, J. and Stevenson, P. (2001) MHC class I ubiquitination by a viral PHD/LAP finger protein. Immunity. **15**, 627-636

25 Coscoy, L., Sanchez, D. and Ganem, D. (2001) A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. J.Cell Biol. **155**, 1265-1273

26 Hewitt, E., Duncan, L., Mufti, D., Baker, J., Stevenson, P. and Lehner, P. (2002) Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation. EMBO J. **21**, 2418-2429

27 Lorenzo, M., Jung, J. and Ploegh, H. (2002) Kaposi's sarcoma-associated herpesvirus K3 utilizes the ubiquitin-proteasome system in routing class major histocompatibility complexes to late endocytic compartments. J.Virol. **76**, 5522-5531

28 Dodd, R., Allen, M., Brown, S., Sanderson, C., Duncan, L., Lehner, P., Bycroft, M. and Read, R. (2004) Solution structure of the kaposi's sarcoma-associated herpesvirus K3 N-terminal domain reveals a novel E2-binding C4HC3-type RING domain. J.Biol.Chem. **279**, 53840-53847

29 Mansouri, M., Bartee, E., Gouveia, K., Hovey Nerenberg, B., Barrett, J., Thomas, L., Thomas, G., McFadden, G. and Fruh, K. (2003) The PHD/LAP-domain protein M153R of myxomavirus is a ubiquitin ligase that induces the rapid internalization and lysosomal destruction of CD4. J.Virol. **77**, 1427-1440

30 Coscoy, L. and Ganem, D. (2000) Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. Proc.Natl.Acad.Sci.U.S.A **97**, 8051-8056

31 Nagase, T., Ishikawa, K., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N. and Ohara, O. (1998) Prediction of the coding sequences of unidentified human genes. IX. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res. **5**, 31-39

32 Patil, C. and Walter, P. (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr.Opin.Cell Biol. **13**, 349-355

33 Travers, K., Patil, C., Wodicka, L., Lockhart, D., Weissman, J. and Walter, P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell **101**, 249-258

34 Hampton, R. (2000) ER stress response: getting the UPR hand on misfolded proteins. Curr.Biol. **10**, R518-R521

35 van Laar, T., Schouten, T., Hoogervorst, E., van Eck, M., van der Eb, A. and Terleth, C. (2000) The novel MMS-inducible gene Mif1/KIAA0025 is a target of the unfolded protein response pathway. FEBS Lett. **469**, 123-131

36 Chau, V., Tobias, J., Bachmair, A., Marriott, D., Ecker, D., Gonda, D. and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science **243**, 1576-1583

37 Shamu, C., Flierman, D., Ploegh, H., Rapoport, T. and Chau, V. (2001) Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. Mol.Biol.Cell **12**, 2546-2555

38 Ishido, S., Wang, C., Lee, B., Cohen, G. and Jung, J. (2000) Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. J.Virol. **74**, 5300-5309

39 Coscoy, L. and Ganem, D. (2001) A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates T cell costimulation. J.Clin.Invest **107**, 1599-1606

40 Ishido, S., Choi, J., Lee, B., Wang, C., DeMaria, M., Johnson, R., Cohen, G. and Jung, J. (2000) Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. Immunity. **13**, 365-374

41 Mandart, E., Dufour, M. and Lacroute, F. (1994) Inactivation of SSM4, a new Saccharomyces cerevisiae gene, suppresses mRNA instability due to rna14 mutations. Mol.Gen.Genet. **245**, 323-333

42 Johnson, P., Swanson, R., Rakhilina, L. and Hochstrasser, M. (1998) Degradation signal masking by heterodimerization of MATalpha2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. Cell **94**, 217-227

43 Hitchcock, A., Auld, K., Gygi, S. and Silver, P. (2003) A subset of membrane-associated proteins is ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery. Proc.Natl.Acad.Sci.U.S.A **100**, 12735-12740

44 Gnann, A., Riordan, J. and Wolf, D. (2004) CFTR Degradation Depends on the Lectins Htm1p/EDEM and the Cdc48 Protein Complex in Yeast. Mol.Biol.Cell **15**, 4125-4135

45 Lu, Z., Xu, S., Joazeiro, C., Cobb, M. and Hunter, T. (2002) The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol.Cell **9**, 945-956

46 Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C. and Fushman, D. (2004) Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. J.Biol.Chem. **279**, 7055-7063

47 Flierman, D., Ye, Y., Dai, M., Chau, V. and Rapoport, T. (2003) Polyubiquitin serves as a recognition signal, rather than a ratcheting molecule, during retrotranslocation of proteins across the endoplasmic reticulum membrane. J.Biol.Chem. **278**, 34774-34782

48 Elkabetz, Y., Kerem, A., Tencer, L., Winitz, D., Kopito, R. and Bar-Nun, S. (2003) Immunoglobulin light chains dictate vesicular transport-dependent and -independent routes for IgM degradation by the ubiquitin-proteasome pathway. J.Biol.Chem. **278**, 18922-18929

49 Finley, D., Sadis, S., Monia, B., Boucher, P., Ecker, D., Crooke, S. and Chau, V. (1994) Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. Mol.Cell Biol. **14**, 5501-5509

50 Kikkert, M., Hassink, G., Barel, M., Hirsch, C., van der Wal, F. and Wiertz, E. (2001) Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. Biochem.J. **358**, 369-377

51 Brown, K., Hostager, B. and Bishop, G. (2002) Regulation of TRAF2 signaling by self-induced degradation. J.Biol.Chem. **277**, 19433-19438

52 Wiertz, E., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T., Rapoport, T. and Ploegh, H. (1996) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature **384**, 432-438

53 Pilon, M., Schekman, R. and Romisch, K. (1997) Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. EMBO J. **16**, 4540-4548

54 Zhou, M. and Schekman, R. (1999) The engagement of Sec61p in the ER dislocation process. Mol.Cell **4**, 925-934

55 Ye, Y., Shibata, Y., Yun, C., Ron, D. and Rapoport, T. (2004) A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature **429**, 841-847

56 Lilley, B. and Ploegh, H. (2004) A membrane protein required for dislocation of misfolded proteins from the ER. Nature **429**, 834-840

57 Bos, E., Dobbe, J., Luytjes, W. and Spaan, W. (1997) A subgenomic mRNA transcript of the coronavirus mouse hepatitis virus strain A59 defective interfering (DI) RNA is packaged when it contains the DI packaging signal. J.Virol. **71**, 5684-5687

58 Hellman, U., Wernstedt, C., Gonez, J. and Heldin, C. (1995) Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal.Biochem. **224**, 451-455