GAS translocation through polarized keratinocyte monolayers

Keratinocyte monolayers on Transwells were inoculated with GAS at a multiplicity of infection of 10 per keratinocyte. Monolayers were incubated at 37 °C with 5% CO_2. The keratinocyte medium in the upper chamber was replaced 2 h after adding GAS, and the Transwell inserts containing the keratinocytes were moved to new wells containing fresh medium every 2 h to prevent overgrowth of translocated bacteria. We assessed translocation by quantitative cultures of medium from the lower chamber at 2 h intervals.

Apligraf living skin equivalent (a gift from Organogenesis) was used as a model for human skin[14]. Samples of skin equivalent were cut into rectangular sections, each approximately 2.5 × 1.2 cm. Each section was placed on a 12 mm Transwell (3.0-μm pore size; Costar) and the Transwell was placed on Todd Hewitt agar containing 5% sheep blood. The apical surface of the skin equivalent was lightly brushed with a sterile cytology brush to abrade the stratum corneum, exposing the underlying epidermal keratinocytes. GAS was resuspended at 10^6 colony-forming units per 10 μl^{-1} in SFM supplemented with 0.3 mM calcium chloride, and inoculated onto the surface of the tissue. After inoculation, we incubated tissue samples at 37 °C with 5% CO_2 and no supplemental humidity. Transwells containing the inoculated tissue samples were transferred to fresh blood agar every 2 h. The blood agar plates were then incubated overnight at 37 °C for enumeration of colony-forming units representing the number of organisms emerging from the basal surface of the tissue.

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The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol

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In eukaryotic cells, incorrectly folded proteins in the endoplasmic reticulum (ER) are exported into the cytosol and degraded by the proteasome[1]. This pathway is co-opted by some viruses. For example, the US11 protein of the human cytomegalovirus targets the major histocompatibility complex class I heavy chain for cytosolic degradation[2]. How proteins are extracted from the ER membrane is unknown. In bacteria and mitochondria, members of the AAA ATPase family are involved in extracting and degrading membrane proteins[3,4]. Here we demonstrate that another member of this family, Cdc48 in yeast and p97 in mammals, is required for the export of ER proteins into the cytosol. Whereas Cdc48/p97 was previously known to function in a complex with the cofactor p47 (ref. 5) in membrane fusion[6–8], we demonstrate that its role in ER protein export requires the interacting partners Ufd1 and Npl4. The AAA ATPase interacts with substrates at the ER membrane and is needed to release them as polyubiquitinated species into the cytosol. We propose that the Cdc48/p97–Ufd1–Npl4 complex extracts proteins from the ER membrane for cytosolic degradation.

We proposed that Cdc48/p97 may have a role in ER protein degradation on the basis of several pieces of indirect evidence. First, Cdc48 binds to Ufd1, Ufd2 and Ufd3 (refs 9–11), which are involved in the degradation of ubiquitin-fusion proteins[12]. Second, a complex of Cdc48, Ufd1 and Npl4 (a protein originally identified in a screen for nuclear transport defects[13]) functions in the proteasome-dependent cleavage of ER membrane proteins that serve as the precursors to two transcription factors (Spt23 and Mga2)[14]. Third, both mammalian p97 and yeast Cdc48 interact with the proteasome and a degradation substrate[15,16]. Finally, a large fraction of both Cdc48 and p97 is associated with ER membrane (ref. 6 and Y.Y., unpublished results).

To test directly the involvement of Cdc48 in ER protein degradation, we analysed the stability of misfolded ER proteins in *CDC48* mutants of *Saccharomyces cerevisiae*. As a membrane protein substrate we choose H–$2K^b$, a major histocompatibility complex (MHC) class I heavy chain that in yeast is quickly degraded in a proteasome-dependent manner[17]. Wild-type or *cdc48-1* mutant cells were pulse-labelled with [^{35}S]methionine at a non-permissive temperature and incubated with unlabelled methionine for different time periods. Cell lysates were subjected to immunoprecipitation with antibodies to H–$2K^b$. The results showed that H–$2K^b$ was much more stable in *cdc48-1* mutant than in wild-type cells (Fig. 1a). A significant stabilization of H–$2K^b$ was seen with another *CDC48* mutant (*cdc48-3*), even at permissive temperature (Fig. 1b).

We tested the misfolded lumenal protein CPY*, an aberrant form of carboxypeptidase Y (CPY), which is degraded by the ubiquitin-proteasome pathway[18]. The stability of CPY* was analysed by immunoblotting after inhibition of protein synthesis with cycloheximide. Whereas CPY* was rapidly degraded in wild-type cells at both 30 °C and 23 °C, the protein was significantly more stable in *cdc48-1* cells (Fig. 1c), particularly at 23 °C—the restrictive temperature for the mutant. The half-life of CPY* was also increased in

cdc48-3 mutant cells (Fig. 1d). Together, these results show that the degradation of both membrane and soluble ER proteins depends on the function of Cdc48.

Next we tested the role of Ufd1 and Npl4, two known interaction partners of Cdc48 (refs 14, 19), in the degradation of ER proteins. In the *ufd1-1* mutant both H–2K^b and CPY* were significantly stabilized (Fig. 2a, c). Similar results were obtained with *NPL4* mutants even at permissive temperature (Fig. 2b; also compare upper and middle row of panels d, e). When mutant cells expressed the wild-type Npl4 protein, rapid degradation of CPY* was restored (Fig. 2d, e, lower rows). These data indicate that Ufd1 and Npl4 also have a role in ER protein degradation. Other Ufd proteins (Ufd2, Ufd4 and Ufd5) do not seem to be involved, as deletion mutants did not show any defects in ER protein degradation (Supplementary Information Fig. 1).

In *CDC48*, *UFD1* and *NPL4* mutants, stabilized CPY* appears to remain inside the ER as indicated by the fact that it remained associated with the membrane and protected from added protease (Supplementary Information Fig. 2). As Cdc48 interacts with Ufd1 and Npl4 (refs 14, 19), these data suggest that the complex is required for the export of misfolded ER substrates. Consistent with the observation that the accumulation of misfolded proteins in the ER generally leads to the induction of the unfolded protein response (UPR)[17], β-galactosidase under the control of a UPR-element-containing promoter was expressed in *cdc48-3*, *ufd1-1* and *npl4-2* mutants, but not in the corresponding wild-type strains (Fig. 2f).

Next we tested whether p97, the mammalian homologue of yeast Cdc48, has a similar role in the export of ER proteins. Retrograde translocation of MHC class I heavy chains, induced by the cytomegalovirus protein US11, was studied in permeabilized astrocytoma cells in the presence of a proteasome inhibitor to prevent subsequent heavy chain degradation in the cytosol[2,20].

To interfere with the function of p97, we generated a dominant negative mutant similar to one used for the trypanosomal homologue[21]. An ATP hydrolysis-defective mutant was constructed by changing the glutamic acid residues at positions 305 and 578 in rat p97—located within the Walker B motifs of the two ATPase domains—to glutamine residues (QQ mutant). Wild-type p97 and the QQ mutant were expressed as His-tagged proteins in *Escherichia coli*, and purified. As expected, the ATPase activity of the mutant p97 was at least tenfold lower than that of wild-type p97, but the interactions with Npl4/Ufd1 and p47 were maintained (Supplementary Information Fig. 3).

Astrocytoma cells expressing US11 were pulse-labelled with [^{35}S]-methionine and permeabilized. Either wild-type or mutant p97 was added at about threefold excess over endogenous protein (Supplementary Information Fig. 4). After a chase incubation, the samples were either analysed directly by immunoprecipitation with anti-heavy-chain antibody, or first fractionated into a pellet fraction (containing the ER membranes) and a supernatant fraction (containing cytosol) before analysis (Fig. 3a). In the absence of added p97, MHC heavy chains are exported from the ER and deglycosylated in the cytosol[2,20]. Accordingly, deglycosylated chains appeared during the chase (lane 2) and fractionated with the cytosol (lane 4),

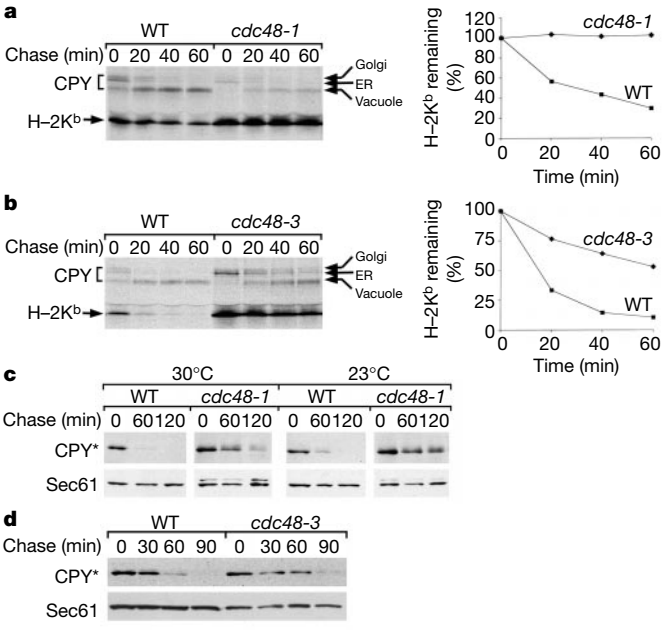


Figure 1 Cdc48 is required for ER protein degradation in *S. cerevisiae*. **a**, Stability of the heavy chain H–2K^b analysed in pulse-chase experiments at 23 °C for wild-type (WT) and *cdc48-1* yeast cells. Transport of carboxypeptidase Y (CPY) from the ER via the Golgi to the vacuole[17] was not affected in the mutant (the different forms of CPY are indicated). The graph shows quantification of the experiment. **b**, Stability of H–2K^b analysed in WT and *cdc48-3* cells at 30 °C. The graph shows the mean of two experiments. **c**, Stability of CPY* analysed in WT and *cdc48-1* cells at the indicated temperatures by immunoblotting after addition of cycloheximide. Immunoblotting with Sec61 antibodies was used to verify equal loading. **d**, Stability of CPY* in WT and *cdc48-3* cells at 30 °C. The same results were obtained when isogenic WT and *cdc48-3* strains, generated by three rounds of backcrossing, were compared, or when oleic acid was added to the growth medium to compensate for potential defects in lipid synthesis of this mutant (not shown).

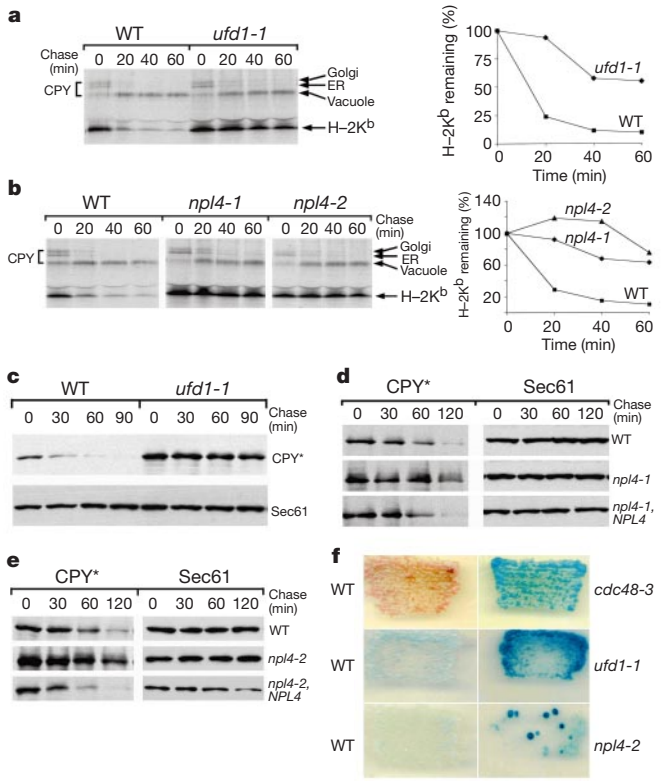
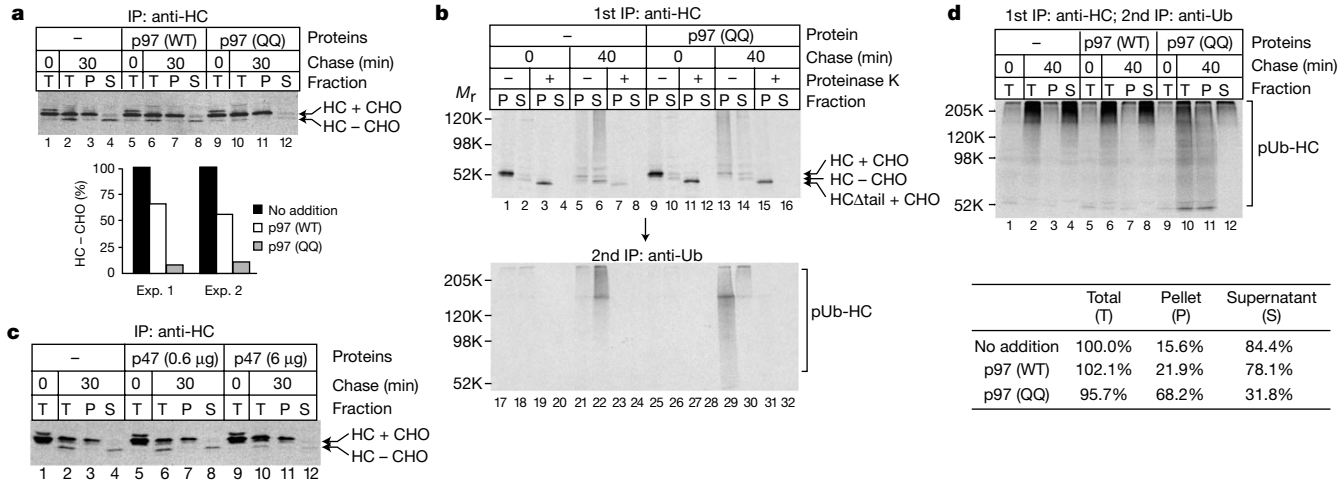


Figure 2 The Cdc48 interacting proteins Ufd1 and Npl4 are required for ER protein degradation. **a**, Stability of H–2K^b analysed by pulse-chase experiments at 25 °C in wild-type (WT) and *ufd1-1* cells. The graph shows the quantification of the experiment. **b**, Stability of H–2K^b in WT, *npl4-1* and *npl4-2* cells. The graph shows quantification of the experiment. **c**, Stability of CPY* analysed in WT and *ufd1-1* cells by immunoblotting after addition of cycloheximide at 30 °C. Immunoblotting with Sec61 antibodies served as a loading control. **d**, Stability of CPY* analysed in WT and *npl4-1* cells. Where indicated, the cells expressed the WT gene *NPL4* on a plasmid. **e**, Stability of CPY* analysed in WT and *npl4-2* cells. **f**, Expression of β-galactosidase under a UPR-element-containing promoter in mutant and corresponding WT strains, visualized by the appearance of blue.



	Total (T)	Pellet (P)	Supernatant (S)
No addition	100.0%	15.6%	84.4%
p97 (WT)	102.1%	21.9%	78.1%
p97 (QQ)	95.7%	68.2%	31.8%

Figure 3 Mammalian p97 is required for export of MHC class I heavy chains from the ER. **a**, US11-expressing cells were labelled, permeabilized and chase-incubated with cytosol alone (–) or addition of wild-type p97 (p97 (WT)) or mutant protein p97 (QQ). They were either analysed directly (T) or first fractionated into membrane pellet (P) and supernatant (S) fractions before immunoprecipitation (IP) with heavy chain (HC) antibodies. HC + CHO and HC – CHO indicate glycosylated and deglycosylatyed HC, respectively. The graph shows the relative amount of deglycosylated heavy chains accumulated during the chase in two experiments. **b**, After the chase incubation as in **a**, proteinase K was added as indicated, and the samples were subjected to immunoprecipitation with heavy chain antibodies (top panel). HCΔtail + CHO, glycosylated HC, the cytoplasmic tail of which has been cleaved off. A portion of the samples was subjected to another round of immunoprecipitation with ubiquitin (Ub) antibodies (lower panel). pUb-HC, polyubiquitinated HC. **c**, As in **a**, with the indicated amounts of p47 added. **d**, Ubiquitinated heavy chains in the membrane and supernatant fractions analysed by double immunoprecipitation as in **b**. The table shows quantification of the ubiquitinated heavy chains at the end of the chase.

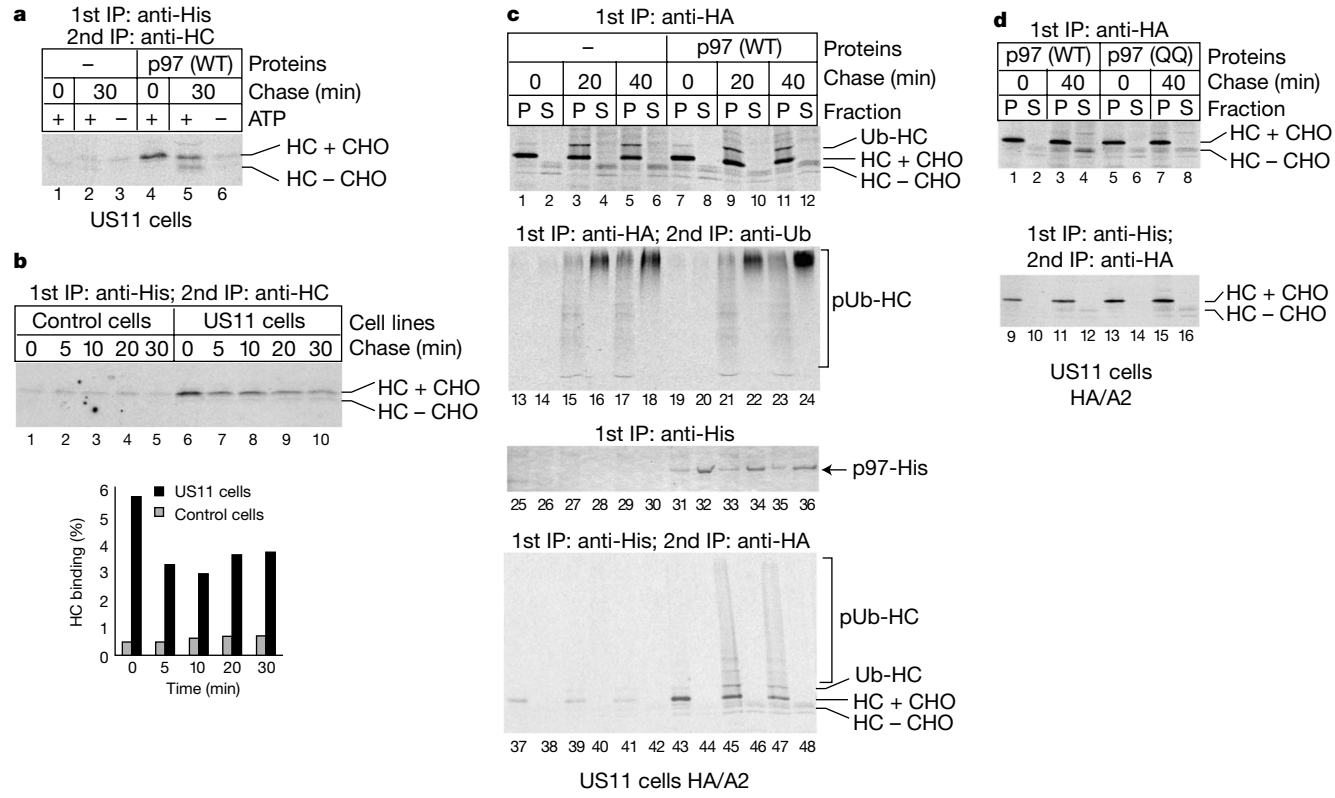


Figure 4 Association of p97 with MHC class I heavy chains. **a**, US11-expressing cells were labelled and permeabilized in the presence or absence of His-tagged wild-type p97 (p97 (WT)). Where indicated, ATP was depleted. The samples were chase-incubated for the indicated time period, solubilized and subjected to sequential immunoprecipitation (IP) with His and heavy chain (HC) antibodies. HC + CHO and HC – CHO indicate glycosylated and deglycosylated HC, respectively. **b**, As in **a**, but with both control and US11-expressing cells. The graph shows the amount of p97-associated heavy chains relative to the total amount, determined in separate immunoprecipitations (not shown). **c**, As in **a**, but with a cell line expressing both US11 and HA-tagged heavy chain (HA/A2). The samples were fractionated into a membrane pellet (P) and cytosol (S) fraction. Ten per cent of each fraction was used for direct immunoprecipitation for heavy chains with HA antibodies (lanes 1–12). Twenty per cent of each fraction was sequentially immunoprecipitated with HA and ubiquitin (Ub) antibodies (lanes 13–24). The remainder of each fraction was immunoprecipitated with His antibodies. An aliquot was analysed by staining with Coomassie blue (lanes 25–36), and the rest was subjected to a second immunoprecipitation with HA antibodies (lanes 37–48). pUb-HC and Ub-HC indicate polyubiquitinated and mono-ubiquitinated HC, respectively. **d**, As in **c**, except that both wild-type (WT) and mutant p97 were used. Ten per cent of the sample was directly immunoprecipitated with HA antibodies (lanes 1–8). The rest of the sample was sequentially immunoprecipitated with His and HA antibodies (lanes 9–16).

whereas the glycosylated chains remained in the ER (lane 3). The addition of wild-type p97 had a moderate effect on retro-translocation of the heavy chain. On the other hand, mutant p97 strongly inhibited the export of heavy chains (Fig. 3a). These chains remained inside the ER as only their cytoplasmic tails were degraded by added protease (Fig. 3b, compare lane 15 and lane 7).

We attempted to inhibit the function of p97 in ER protein degradation by addition of p47, the cofactor required for membrane fusion[5]. This protein competes with Ufd1 and Npl4 for p97 binding[19] and decreases the ATPase activity of p97 (ref. 22). We found that addition of purified recombinant p47 to permeabilized cells inhibited retro-translocation of MHC heavy chains (Fig. 3c).

Next we tested the effect of mutant p97 and of p47 on the polyubiquitination of MHC heavy chains, a process that precedes the release of the chains into the cytosol[20,23]. The material that was immunoprecipitated with heavy chain antibodies was subjected to a second round of immunoprecipitation with ubiquitin antibodies to enrich for the small fraction of ubiquitinated heavy chains. In the absence of added p97, polyubiquitinated chains accumulated during the chase period and appeared in the cytosol fraction. Wild-type p97 had little effect on polyubiquitination and the release of these chains from the ER membrane. In contrast, the addition of mutant p97 inhibited the release of ubiquitinated chains into the cytosol but did not affect the total amount of ubiquitinated heavy chains (Fig. 3d). The heavy chains accumulating on the membrane carried fewer ubiquitin molecules than those in the cytosol (Fig. 3b, d), suggesting that long ubiquitin chains are required for release of the substrate from the membrane. As expected, the ubiquitinated chains on the membrane were sensitive to protease treatment (Fig. 3b). The release of polyubiquitinated heavy chains into the cytosol was also inhibited by the addition of p47 (Supplementary Information Fig. 5).

Finally, we investigated whether p97 associates with substrate. His-tagged p97 protein was added to labelled permeabilized cells, the membranes were solubilized in a mild detergent, and p97 was precipitated with anti-His antibodies. A significant proportion of the MHC heavy chains was co-precipitated, even at the beginning of the chase period, as demonstrated by a second immunoprecipitation (Fig. 4a, lanes 4 and 5). Co-precipitation was reduced when ATP was depleted (lane 6). In the absence of added p97 very little material was co-precipitated (lanes 1–3). Similar results were obtained with cells that express haemagglutinin (HA)-tagged MHC heavy chains, using HA antibodies in the second immunoprecipitation[20] (Supplementary Information Fig. 6). The association between p97 and MHC heavy chains was much more pronounced in US11-expressing cells than in control cells (Fig. 4b, lanes 6–10 compared with 1–5; see also quantification graph). Binding was not detected if p97 was added after solubilization of the membranes (Supplementary Information Fig. 7). Cell fractionation showed that p97 associated preferentially with heavy chains on the membrane (Fig. 4c, lanes 43–48), despite the fact that most of the added p97 was cytosolic (Fig. 4c, lanes 31–36). Polyubiquitinated heavy chains were much less abundant on the membrane than in the cytosol (Fig. 4c, lanes 21–24), yet only the ones on the membrane, carrying fewer ubiquitin molecules, were found in association with p97 (Fig. 4c, lanes 45 and 47). Although the ATP hydrolysis mutant of p97 did not support release of substrate into the cytosol, it did interact with heavy chains (Fig. 4d).

Taken together, our data indicate that the Cdc48/p97–Ufd1–Npl4 complex is involved in ER protein degradation in yeast and mammals. The AAA ATPase binds the substrate on the ER membrane and releases it into the cytosol on ATP hydrolysis. Compromising the function of Cdc48/p97 leads to the accumulation of substrates in the ER lumen, although a small portion is found as polyubiquitinated molecules on the membrane. Exactly how ubiquitination cooperates with Cdc48/p97-driven substrate release remains to be clarified. Although p97 can recognize ubiquitin[24], we found that the ATPase was associated with both unmodified and modified MHC class I heavy chains, suggesting that p97 binds substrates either before or during polyubiquitination. On the basis of our data, we propose that Cdc48/p97 is involved in extracting proteins from the ER membrane. This function would be similar to that of other members of the AAA family of ATPases, which contain an additional protease domain (AAA proteases). FtsH in *E. coli* and AAA proteases in mitochondria appear to grab a misfolded membrane protein at one terminus and sequentially pull it out for degradation[3,4]. The structure of p97 indicates that there are two hexameric rings stacked on top of each other with a pore in the middle[25]. One possibility is that the Cdc48/p97 ring is bound to the ER membrane and pulls the polypeptide chain through its pore in an ATP-dependent manner. This would be analogous to the mechanism by which hexameric helicases move along single-stranded nucleic acids[26], or by which the hexameric ATPase rings move polypeptides into the associated proteolytic chambers of the eukaryotic proteasome or the bacterial ClpP[27]. □

Methods

Strains and plasmids

The wild-type strains used in this study were: W303α (*MATα, his3-11, trp1-1, ura3-1, ade2-1, leu2-3,-112, can1-100*); BWG1-7a (*MAT**a**, his4-519, ura3-52, ade1-100, leu2-3,-112*), provided by E. Johnson as an isogenic strain for *ufd1-1*; S288C (*MAT**a**, ura3-52, leu2Δ1, trp1Δ63*), provided by P. Silver as an isogenic strain for *npl4-1* and *npl4-2*; and BY4742 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*), obtained from Research Genetics as an isogenic strain *for Δufd2, Δufd3, Δufd4, Δufd5*. MLY1763 (*ade2-101, lys2-801, ura3-52, cdc48-1*), PSY2340 (*MAT**a**, ura3-52, leu2Δ1, trp1Δ63, npl4-1*), PSY2341 (*MATa, ura3-52, leu2Δ1, trp1Δ63, npl4-2*) and the rescue plasmid pPS402 containing the *NPL4* gene were a gift from P. Silver. WPY106 (*MAT**a**, ura3-52, leu2-3, cdc48-3*) was provided by W. Prinz. We backcrossed this strain three times to W303α to obtain an isogenic pair. PM373 (*MAT**a**, his4-519, ura3-52, ade1-100, leu2-3,-112, ufd1-1*) was provided by E. Johnson. Strains 13888 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Δufd2*), 15063 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Δufd3*), 14859 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Δufd4*) and 13716 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Δufd5*) were obtained from Research Genetics. The pTS-A2 plasmid expessing H–2K^b was provided by H. Ploegh, and the pRS306-*prc1-1* plasmid encoding CPY* was a gift from T. Sommer. The pMCZ-Y plasmid containing the UPRE-lacZ reporter was provided by R. Schekman.

Antibodies

Antibodies to MHC class I heavy chain, ubiquitin and HA (12CA5) have been described previously[20], as have those to CPY[17] and to Kar2 and Sec61 (ref. 28). The anti-Cdc28 antibody was a gift from N. Bouquin and R. Li[29]. The His and p97 antibodies were purchased from Qiagen and Research Diagnostics, respectively.

Pulse-chase labelling and cycloheximide chase analysis

Pulse-chase experiments and immunoprecipitations were performed essentially as described[17]. Cells were grown at 30 °C to mid-log phase, and were labelled for 10 min at the same temperature or shifted to non-permissive temperature for 20 min before labelling. For cycloheximide chase experiments, cells with an absorbance of 10.0 at 600 nm were resuspended in 2.5 ml YPD medium containing 100 μg ml^{-1} cycloheximide. Samples were taken at the different time points and proteins were prepared for immunoblotting as described[12].

Cellular fractionation and protease protection experiments

Cells with an absorbance of 25 at 600 nm were taken at the indicated time points during cycloheximide chase. Spheroplasts were made and homogenized as described[30]. Aliquots of cell lysates were centrifuged for 10 min at 20,000*g* in a microcentrifuge, and the resulting pellet and supernatant fractions were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Parallel aliquots were treated with 0.5 mg ml^{-1} proteinase K on ice for 30 min either in the absence or presence of 1% Triton X-100. We added 20 mM phenylmethylsulphonyl fluoride (PMSF) to inhibit proteinase K before SDS–PAGE analysis.

Purification and characterization of p97

The p97 mutant was generated by mutating codons E305 and E578 to Q in plasmid pQE-p97 (ref. 19) using the Stratagene Quickchange method. The sequence was confirmed. Rat liver p97 and recombinant p97 were purified as described[5,19]. Binding experiments of p97 to glutathione *S*-transferase fusion proteins were carried out as before[19]. We measured the ATPase activity of p97 as described[22].

Experiments with mammalian cells

The experiments were essentially performed as described[20]. Briefly, astrocytoma cells

expressing US11 were incubated for 5 min in the presence of 50 μM proteasome inhibitor ZL_3VS and 27.5 μCi [^{35}S]methionine per 1.0×10^6 cells. After washing, the cells were permeabilized by addition of 0.028% digitonin on ice in the presence of an ATP regenerating system, and unless otherwise indicated, 6 μg p97 or p47 was added for 30 min on ice. The samples were then incubated at 37 °C for different time periods, and separated into membrane and supernatant fractions. Immunoprecipitation with heavy chain antibodies and re-precipitation with ubiquitin antibodies were done as described[20]. When ubiquitinated chains were analysed, deubiquitination was inhibited by addition of 20 μM ubiquitin and 1 μM ubiquitin aldehyde during the chase. With the exception of the experiment in Fig. 3d, 2 mM *N*-ethylmaleimide (NEM) was added during sample preparation. To deplete ATP, the ATP regenerating system was omitted and 0.2 U μl^{-1} hexokinase and 50 mM glucose were added. To analyse p97-associated MHC heavy chains, cell fractions were solubilized in 1% deoxyBig CHAP, 30 mM Tris/HCl pH 7.4, 150 mM potassium acetate, 4 mM magnesium acetate, 1 mM ATP and protease inhibitors. Immunoprecipitation was carried out with His antibodies followed by a second precipitation with heavy chain or HA antibodies. For protease protection experiments, 0.35 mg ml^{-1} proteinase K was added to the fractions and the incubation was carried out for 15 min on ice before addition of 10 mM PMSF.

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phot1 and phot2 mediate blue light regulation of stomatal opening

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The stomatal pores of higher plants allow for gaseous exchange into and out of leaves. Situated in the epidermis, they are surrounded by a pair of guard cells which control their opening in response to many environmental stimuli, including blue light[1,2]. Opening of the pores is mediated by K^+ accumulation in guard cells through a K^+ channel and driven by an inside-negative electrical potential[3]. Blue light causes phosphorylation and activation of the plasma membrane H^+-ATPase that creates this potential[1,2,4–6]. Thus far, no blue light receptor mediating stomatal opening has been identified[7], although the carotenoid, zeaxanthin, has been proposed[2,8]. *Arabidopsis* mutants deficient in specific blue-light-mediated responses have identified[7,9–14] four blue light receptors, cryptochrome 1 (cry1), cryptochrome 2 (cry2), phot1 and phot2. Here we show that in a double mutant of phot1 and phot2 stomata do not respond to blue light although single mutants are phenotypically normal. These results demonstrate that phot1 and phot2 act redundantly as blue light receptors mediating stomatal opening.

Blue light responses of higher plants include de-etiolation, phototropism, chloroplast relocation, and stomatal opening[7]. cry1 and cry2 mediate de-etiolation[9], and phot1 and phot2 (nph1 and npl1, respectively[10]) mediate phototropism[11,12] and chloroplast relocation[12–14]. These blue-light-mediated responses allow plants to capture light energy efficiently, or to avoid damage from high light intensity, thereby optimizing photosynthesis and growth under various light conditions. Photosynthesis is regulated by stomatal aperture.

We considered the phot1 protein and its homologue phot2 as candidates for stomatal blue-light receptors because of their structural properties. The phot1 protein is a serine/threonine protein kinase[11] and mediates the increase in cytosolic Ca^{2+} in response to blue light[15]. A serine/threonine protein kinase[6] and an increase in cytosolic Ca^{2+} (refs 2, 16) have both been reported to be involved in