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Research Article

Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis

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Abstract

Recent studies have identified Derlin-1, a protein that associates with the AAA-ATPase p97 and is implicated in late steps in ER-associated protein degradation (ERAD). Derlin-1 has two *Saccharomyces cerevisiae* homologues, Der1p and Dfm1p. While Der1p has been studied extensively, little is known about Dfm1p. Accordingly, we investigated the role of Dfm1p in ERAD, ER homeostasis and interactions with the yeast p97 homologue Cdc48p. Dfm1p was not involved in the degradation of a number of Der1-dependent or -independent ERAD substrates, neither was it redundant with either Der1p or Sec61p in ERAD. However, Dfm1p had a role in ER homeostasis, since Dfm1p loss or overexpression could stimulate the unfolded protein response (UPR). Furthermore, Dfm1p interacted both genetically and physically with Cdc48p, the yeast p97 homologue, and this interaction required an eight amino acid sequence found in the C-terminus of Dfm1p that we have termed the SHP box. Our genetic studies are consistent with the lack of a role for Dfm1p in ERAD, but indicate it participates in ER-related Cdc48p actions distinct from retrotranslocation. Finally, sequence analysis indicated that the UPR-related and Cdc48p interaction functions of Dfm1p could be separated, implying this protein probably has numerous actions in the cell. Thus, the interaction between Derlins and p97 is conserved between yeast and mammals, although its function in ERAD is not. Furthermore, Dfm1p interacts with Cdc48p through its SHP boxes, and so defines a new motif for interaction with this widely-employed AAA-ATPase.

Keywords: Derlin; CDC48; p97; ERAD; UPR; retrotranslocation; homotypic fusion; UFE1

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Introduction

ER-associated degradation (ERAD) refers to the ubiquitin-mediated degradation of both lumenal and integral membrane proteins of the endoplasmic reticulum. This process is conserved from yeast to mammals and is responsible for the destruction of diverse proteins that are often misfolded, unassembled or damaged. Many ERAD substrates have some or all of their sequence in the ER lumen, and these portions must be moved across the ER membrane in order for ubiquitin-mediated proteasomal hydrolysis to occur. This movement of proteins from the lumen to the cytosol is generally referred to as 'retrotranslocation' or 'dislocation', to distinguish it from the anterograde movement of cytoplasmically synthesized proteins into the lumen that is a normal part of ER protein kinesis.

One of the large open questions concerning ERAD is the mechanism for substrate retrotranslocation. Numerous studies have implicated the hexameric AAA-ATPases, mammalian p97 and its yeast homologue, Cdc48p, in this step in their respective organisms, possibly supplying

the motive force for protein extraction from the ER (Bays *et al*., 2001; Ye *et al*., 2001, 2003). p97/*CDC48* has been reported to be involved in ERAD in a number of different species for substrates that are processed by distinct ligases (Bays *et al*., 2001; Huyer *et al*., 2004; Ye *et al*., 2001). In addition, it is reasonably presumed that a protein pore mediates the actual transport of ERAD substrates out of the lumen, in a manner analogous to that used to move proteins across membranes in traditional secretion. Although some studies have suggested a role for the anterograde channel *SEC61* (Plemper *et al*., 1999; Wiertz *et al*., 1996), a new candidate family of proteins called the Derlins has received significant attention. This class of proteins was independently discovered by two groups exploring virus-mediated ER degradation of MHC class I (Lilley and Ploegh, 2004; Ye *et al*., 2004). Derlin-1 is a small multi-spanning membrane protein required for MHC-I degradation, and is named for its homology with yeast Der1p, one of the first ERAD factors characterized (Knop *et al*., 1996). In MHC-I degradation, Derlin-1 appears to function after ubiqutination and forms a complex with mammalian p97. These features led the co-discoverers to propose that this protein may form or be part of the pore by which p97-mediated retrotranslocation proceeds (Lilley and Ploegh, 2004; Ye *et al*., 2004).

Mammalian Derlins have homology to the prototype yeast ERAD factor Der1p (Figure 1A; Lilley and Ploegh, 2004; Ye *et al*., 2004). Like the Derlins, this small protein has multiple membrane spans and resides in the ER. Despite numerous studies involving Der1p (Hitt and Wolf, 2004; Knop *et al*., 1996; Vashist and Ng, 2004), the function of this protein is still unknown, but the connection with Derlin-1 leads to the idea that it is an integral participant in retrotranslocation. If Der1p were in fact a retrotranslocation factor, one would expect it to operate in concert with Cdc48p, which is known to function in retrotranslocation. As a retrotranslocation factor, Cdc48p is a universal degradation requirement, while Der1p functions in ERAD of a subset of substrates. However, Der1p is not the only yeast Derlin protein. There is a second homologue of Derlin-1 in the yeast genome, called *DFM1* (Hitt and Wolf, 2004), for Der1-like family member 1 (Figure 1B). One proposed model is that Dfm1p and Der1p operate together to carry out the

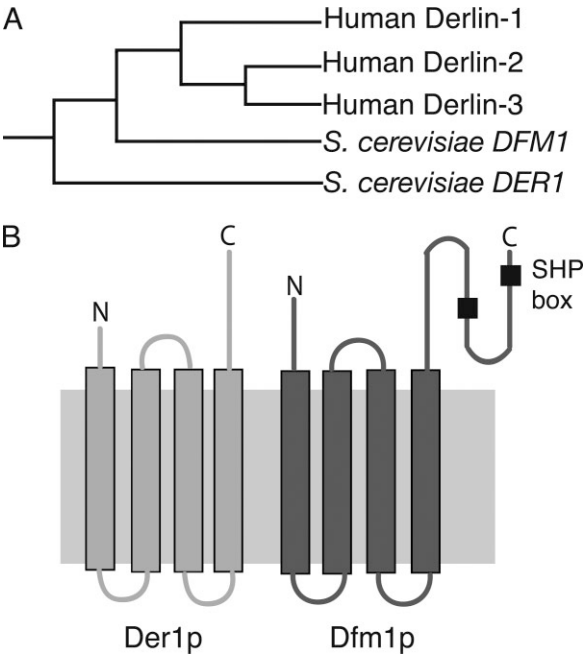

Figure 1. (A) Phylogenetic tree of the human and yeast Derlin homologues. *DFM1* is more closely related to Derlin-1 than its yeast orthologue *DER1*. The tree was constructed as described by Lilley and Ploegh (Lilley and Ploegh, 2004). B) Models of Der1p and Dfm1p. Der1p and Dfm1p are both ER-localized membrane proteins with four transmembrane domains (Hitt and Wolf, 2004). Dfm1p has an extended cytoplasmic tail which contains two 8 amino acid sequences that we have termed SHP boxes

Derlin-1 function in yeast (Lord *et al*., 2005; Schuberth and Buchberger, 2005). Dfm1p is similar to Der1p, and has significant (in fact, slightly higher) homology to mammalian Derlin-1. Unlike Der1p, Dfm1p has an extended C-terminal cytoplasmic tail that contains two eight-amino acid sequences that we have termed SHP boxes (Hitt and Wolf, 2004). These sequences are found in a number of proteins that interact with Cdc48p, including Shp1p and Ufd1p. This leads to the appealing idea that perhaps Der1p and Dfm1p together mediate all ERAD related to Cdc48p. We conducted a study of the Dfm1p protein, with an interest in evaluating its role in the ERAD of a number of substrates, its independence or redundancy with Der1p, its interaction with Cdc48p, and its involvement in ER homeostasis.

We have found that Dfm1p plays a role in the maintenance of ER homeostasis. Furthermore, Dfm1p interacts with Cdc48p both genetically and

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physically, and we have demonstrated that the SHP boxes, which appear to be novel Cdc48p binding sites, mediate this interaction. The effects of Dfm1p on ER stress and Cdc48 can be separated by molecular biological means. However, despite the roles in ER stress and the interaction with Cdc48p, Dfm1p did not function in ERAD by any measure we applied. Thus, Dfm1p's interaction with Cdc48p and role in ER homeostasis are distinct from ERAD, and will lead to a better understanding of the multiple functions of Cdc48 and the Derlins in the cell.

Materials and methods

Plasmids and DNA methods

Polymerase chain reaction (PCR) reactions were performed as follows: Vent DNA polymerase (New England Biolabs, Ipswich, MA) was used in 100 l reactions (1× thermopol buffer, 400 ng template, 1 M each oligo, 2% DMSO, 25 mM Mg, 200 M dNTPs). The PCR reaction was carried out at 94 °C for 2 min followed by 30 cycles of 94 °C for 35 s, 55 °C for 40 s and elongation at 72 °C for varying times, depending on product length. The reaction ended with a 7 min incubation at 72 °C. Oligo sequences used for PCR are available upon request. A table of all plasmids including the figures that feature them is available in the supplementary material (Table S2). All plasmids were constructed with standard molecular biology techniques, as have been described by Gardner *et al*. (1998). The splicing by overlap elongation (SOEing) PCR technique used therein was adapted from Horten *et al*. (1989). The ProteinA-CDC48 plasmid (pRH2078) was a gift from M. Latterich (McGill University, Quebec). Plasmids expressing KHN (pRH1958) and KWW (pRH1960) were a gift from D. Ng (National University of Singapore). The Ste6-166 plasmid (pRH2058) was a gift from S. Michaelis (Johns Hopkins School of Medicine, MD).

Yeast and bacterial strains

Escherichia coli DH5α were grown at 37 °C in LB medium with ampicillin (100 g/ml). Yeast strains were grown at 30 °C, unless otherwise noted, in minimal medium supplemented with dextrose and amino acids, as previously described (Hampton and Rine, 1994). The LiOAc method was utilized to transform yeast strains with plasmid DNA (Ito *et al*., 1983). Null alleles with coding regions replaced by selection markers were constructed by transforming yeast with the LiOAc method with a PCR product that encoded either G418 resistance or CloNAT/nourseothricin (Werner BioAgents, Jena, Germany) resistance and 5′ and 3′ 50 bp flanks homologous to the gene to be disrupted (Baudin *et al*., 1993). Cells were allowed to grow on yeast peptone dextrose (YPD) for ~12 h and then replica-plated onto YPD + 500 g/ml G418 or 200 g/ml nourseothricin. A table of all strains, including genotypes, and the figures that feature them is available in the supplementary materials (Table S1). The *der1Δ* (RHY3604), *dfm1Δ* (RHY3689) and *der1Δdfm1Δ* (RHY3690) strains, as well as the corresponding wild-type strain (RHY3688), were obtained through sporulation of strains 24 247 and 23 341 from the yeast deletion collection (ResGen/Invitrogen, Carlsbad, CA). Haploids of *der1Δ* and *dfm1Δ* strains were then crossed, sporulated and dissected to obtain the double mutant. Gene knock-out was confirmed through PCR. These strains were used for all substrate degradation studies as well as the indicated unfolded protein response flow cytometry experiments. *DER1* or *DFM1* were also disrupted in RHY471, an S288C derivative as described above. The *ufe1-1* and an isogenic wild-type strain were a gift from H. Pelham (MRC Laboratory of Molecular Biology, Cambridge, UK).

Degradation assays and UPR measurements

Cycloheximide chase degradation assays were performed as previously described (Gardner *et al*., 1998). Briefly, yeast strains were grown to log phase (<0.5 OD ABS = 600) and cycloheximide was added to a final concentration of 50 g/ml. At each time point, a constant volume of culture was removed and lysed. Lysis began with the addition of 100 l SUME (1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, 10 mM EDTA) with protease inhibitors and 100 l glass beads, followed by vortexing for 3 min, and finally the addition of 100 l 2× USB (75 mM MOPS, pH 6.8, 4% SDS, 200 mM DTT, 0.2 mg/ml bromophenol blue, 8 M urea), followed by a 10 min incubation at 55 °C. The resulting lysate was clarified by centrifugation and used for SDS–PAGE and immunoblotting. Flow cytometry for GFP was also performed

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as described (Cronin and Hampton, 1999). Cells were grown to OD<0.2. Data was obtained through a FACScalibur machine (Becton, Dickinson and Company, Franklin Lakes, NJ) and statistical analysis was performed with CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ).

Dilution assays

All strains were grown to OD < 0.5 in supplemented minimal medium. A total of 0.35 OD units were then harvested and resuspended in 1 ml sterile water. Five-fold dilutions were then performed, and the serially diluted cultures were spotted on medium with the appropriate supplements to select for plasmids in strains. Plates were then grown at indicated temperatures for 3 days.

Co-immunoprecipitation assays

The protocol was modified from that used by Grandi *et al*. (1993). Strains were grown to OD < 0.5 OD, and 10 OD units were then harvested by centrifugation. Microsomes were made according to Shearer and Hampton (2004). Briefly, cells were resuspended in 1.5 ml diluted XL buffer (XL buffer:1.2 M sorbitol, 5 mM EDTA, 0.1 M KH_2PO_4, pH to 7.5; diluted 1 : 4 in water) with protease inhibitors [4-2(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), tosylphenylalanine chloromethyl ketone (TPCK), leupeptin, pepstatin]. An equal volume of glass beads was added and the resulting mixture was vortexed for 6 × 1 min. The supernatant was then removed from the beads and the beads were washed once with 1 ml of the same diluted XL buffer that was added to the supernatant. The supernatant was then centrifuged for 30 min at 21 000 × *g*. At this point, the microsome pellets were solubilized with an IP buffer containing a non-denaturing detergent to use in co-immunoprecipitation. A total of 300 l of this lysis buffer (2% Triton X-100, 20 mM NaCl, 0.2 mM $MgCl_2$, 20 mM Tris–HCL, pH 8.0) and 100 l glass beads were added to the microsomes, followed by 3 × 1 min of vortexing. The supernatant was removed to a new tube and the beads were washed once with IP buffer (15 mM Na_2HPO_4, 150 mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 mM EDTA, final pH 7.5) with protease inhibitors. The supernatant was spun for 5 min at 16 000 × *g* to remove debris and then incubated with 100 l IgG-Sepharose beads (Amersham Biosciences) in IP buffer (10% w/v) at 4 °C for 1 h, followed by two 1 ml washes with TST buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.5% Tween-20), one 1 ml wash with lysis buffer and two 0.5 ml washes with 5 mM NH_4Ac, pH 5.0. Each wash was followed by a 10 s spin at <100 × *g*. The beads were aspirated to dryness and then 100 l 2× USB was added, followed by a 10 min incubation at 55 °C.

Results

Often, the loss of proteins involved in ER quality control causes an elevation of the unfolded protein response (UPR) (Friedlander *et al*., 2000; Travers *et al*., 2000), a signal transduction pathway activated upon the accumulation of unfolded proteins in the ER (Patil and Walter, 2001). We first examined whether loss of the yeast Derlin Dfm1p would activate this signalling pathway, using an integrating version of the sensitive UPRE4–GFP reporter (Bays *et al*., 2001). This reporter is activated by the UPR, allowing measurement of pathway activity by flow cytometry. Loss of Der1p elevated the UPR, while loss of Dfm1p alone did not cause a detectable change. Each of these results has been reported previously (Hitt and Wolf, 2004; Knop *et al*., 1996). However, we found that addition of a *dfm1Δ* null mutation to an otherwise isogenic *der1Δ* strain produced a further elevation of the UPR above that caused by the *der1Δ* alone (Figure 2), indicating that Dfm1p had a hitherto undetected role in ER homeostasis. This experiment was also performed in the presence of 2 mM DTT, which caused a strong upregulation of UPR, to ensure that each strain was fully proficient in UPR activation (DNS).

Since loss of *DFM1* results in an increased UPR in a *der1Δ* strain, we felt compelled to further test whether Dfm1p participates in ERAD, extending the work of Hitt and Wolf (2004) to a variety of substrates and genetic circumstances. As expected from that work, the loss of Dfm1p had no effect on the degradation of two soluble Der1p-dependent substrates, CPY* and KHN, either alone or with a *der1Δ* (Figure 3A, B). This was also true of the membrane-spanning Der1p-dependent substrate KWW (Figure 3C). We further examined the effect of the *dfm1Δ* mutation on ERAD of two

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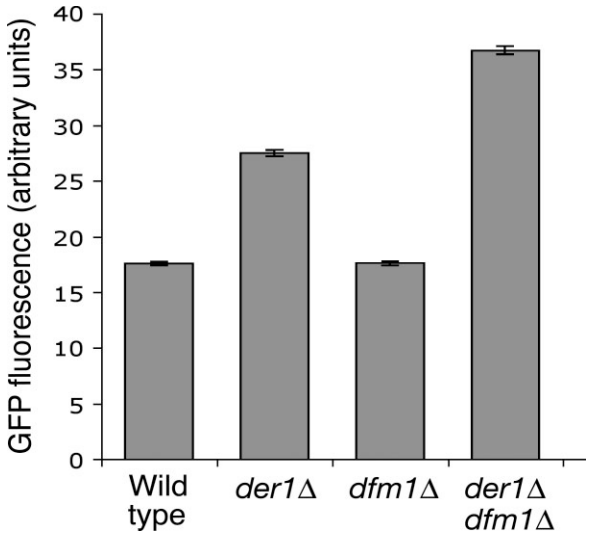

Figure 2. Elevated UPR caused by a *dfm1Δ* null allele relative to wild type and *der1Δ*. The indicated strains expressing the UPRE4-GFP reporter were grown in minimal media and the log phase GFP fluorescence was measured by flow cytometry. In this and subsequent flow cytometry experiments, 10 000 cells were analyzed for each experimental condition. Bars indicate the standard error of the mean

Der1p-independent substrates, Hmg2p and Ste6-166, both alone and in combination with the *der1Δ* mutation. The presence of the *dfm1Δ* had no effect on the degradation of either of these substrates alone or in conjunction with *der1Δ* (Figure 3D, E), as also shown for Ste6-166 in recent work by Kreft *et al*. (2006).

The UPR experiment in Figure 2 indicated that Dfm1p may have functions that are redundant with Der1p, since its loss only had a discernable effect when *DER1* was also absent. Thus, we wondered if Dfm1p might be redundant for Der1p's ERAD function when expressed at sufficient levels. To test this, we made a Dfm1p plasmid with a strong *TDH3* promoter on a 2 plasmid to examine the effects of overexpressing this gene product. Despite the fact that this plasmid causes highly elevated levels of Dfm1p as indicated by immunoblotting (DNS) and had clear phenotypes (see below), there was no suppression of the *der1Δ* as measured by CPY* stabilization (Figure 3F).

It has been hypothesized that the Derlins may be retrotranslocation factors that provide an exit route out of the ER. The translocon Sec61p has also been proposed to mediate transfer of ERAD substrates from the lumen to the cytosol. However, strains lacking *DFM1* and *DER1* do not stabilize several ERAD proteins (see Figure 3) and a *sec61-2* mutant has only minor effects on the degradation of a number of substrates, including Hmg2p. One possibility is that yeast Derlins and Sec61 mediate separate but overlapping exit strategies. Thus, we tested whether the presence of the *der1Δ dfm1Δ* mutation would have an enhancing effect on the minor ERAD defect caused by the *sec61-2* mutation. In a *sec61-2* strain, the degradation of Hmg2p–GFP is slowed by a maximum of two-fold. This small difference is reproducible but cannot be enhanced by preincubation of the mutant cells at the non-permissive temperature for several hours prior to starting the degradation time course (DNS). However, stabilization of the ERAD substrate was not enhanced, and in fact the degradation of Hmg2p-GFP was slightly faster in the *sec61-2 der1Δ dfm1Δ* triple mutant (Figure 4).

While these data indicate that Dfm1p does not participate in ERAD, either independently or in a redundant manner with Der1p or Sec61p, we found clear evidence that the Dfm1p protein plays a role in ER stress and homeostasis (Figure 2). In order to follow up those observations, we first examined whether there were phenotypes associated with overexpressing Dfm1p. To our surprise, this reliably caused a significant increase in UPR signalling, as measured with the UPRE4–GFP reporter (Figure 5A). In contrast, Der1p caused minimal activation of the UPR response when expressed in the same manner, indicating that the UPR phenotype was specific for the Dfm1p protein. Generally, we have not observed any UPR induction upon strong expression of membrane proteins such as 6myc-Hmg2p (Hampton *et al*., 1996) or Hmg2p. With the thought that Dfm1p might cause the UPR effect by somehow interfering with ERAD, we also tested the effect of overexpressing Dfm1p in a *hrd1Δdoa10Δ* double mutant, which inhibits all known ERAD pathways. In this background, the Dfm1p protein was still able to strongly stimulate the UPR above the elevated background caused by loss of these two ERAD ubiquitin ligases (Figure 5B).

Because the Derlins are thought to work with p97, we next evaluated both genetic and physical interactions between Dfm1p and the yeast p97 homologue, Cdc48p. We found that overexpression of Dfm1p has very specific deleterious effects on a

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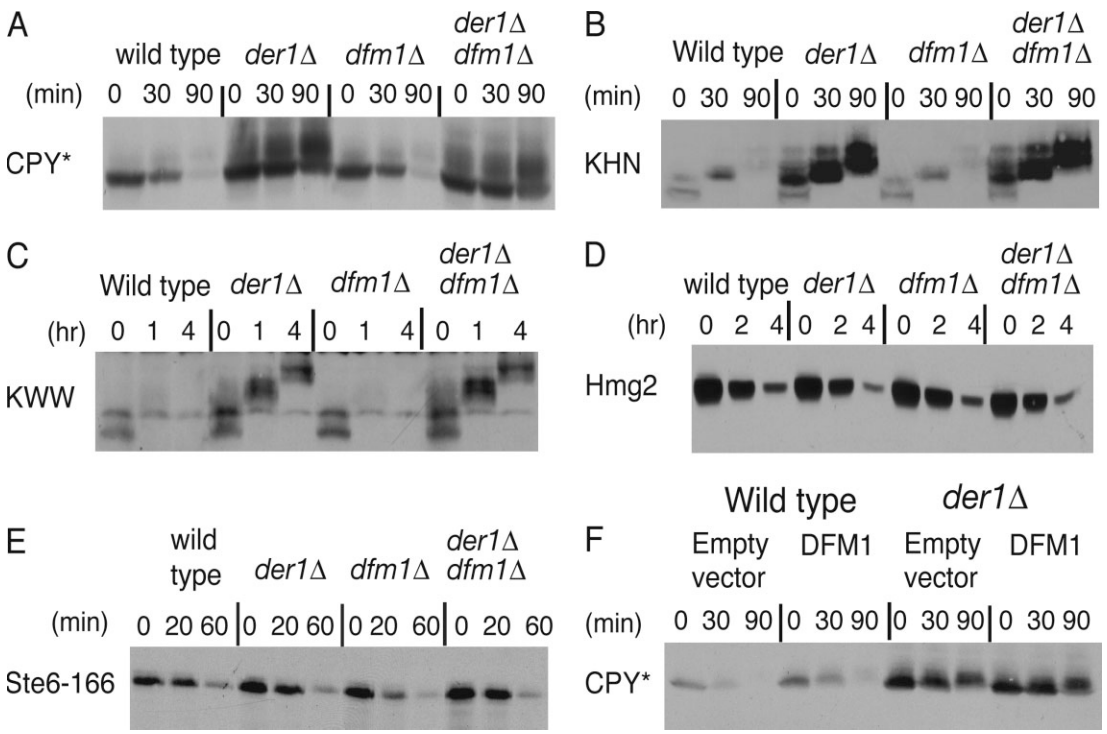

Figure 3. *DFM1* had no role in the degradation of *DER1* dependent or independent ERAD. (A-E) Degradation of the indicated tagged ERAD substrates was measured by cycloheximide chase (CHX) in isogenic strains. After CHX addition, cells were lysed and analyzed by SDS-PAGE, immunoblotting for each substrate. (F) Overexpression of *DFM1* did not suppress a *der1Δ* mutant. Both wild type and *der1Δ* strains containing an empty vector plasmid or overexpressing *DFM1* plasmid were tested for CPY* degradation by cycloheximide chase

cdc48 mutant (Figure 6A). As shown in a dilution plating assay, when Dfm1p was strongly expressed, *cdc48-3* cells had an obvious growth defect at their normally permissive temperature that did not occur in an isogenic wild-type strain. This effect was only observed with the Dfm1p protein; similar expression of Der1p had no effect above that of the empty vector. The phenotype was not specific for the *cdc48-3* allele, as overexpression of Dfm1p had similar effects on a *cdc48-2* strain (DNS). However, the Dfm1-caused growth defect was also highly specific for *cdc48* loss of function and was not a general phenotype for temperature-sensitive strains. Dfm1p overexpression in a temperature-sensitive mutant of *cdc34*, a cell cycle protein not related to ERAD, had no effect (DNS).

We next asked whether this Dfm1p overexpression phenotype was similarly observable in an *npl4* mutant. Npl4p, along with a second co-factor, Ufd1p, binds to Cdc48p to form a complex which functions in ERAD. These proteins are functionally related, as a mutation in any of the three produces similar ERAD defects (Bays *et al*., 2001). We reasoned that a similar genetic interaction would be evident between *DFM1* and *NPL4*. Surprisingly, the presence of overexpressed Dfm1p in an *npl4* mutant did not result in the killing phenotype observed with the *cdc48* mutant (Figure 6B). Even when the overexpression experiment was performed only 2 °C below the non-permissive temperature, addition of Dfm1p had no effect on the *npl4* mutant growth (Figure 6B). Thus, it appears that the Dfm1p protein shows a very specific genetic interaction with Cdc48p. This action of Dfm1p appears to be unrelated to the Cdc48p–Npl4p–Ufd1p complex, as similar effects were not observed between Dfm1p and an *npl4-1* mutant.

Cdc48p has numerous cellular functions (Bays *et al*., 2001; Cao *et al*., 2003; Latterich *et al*., 1995). The above data indicate that Dfm1p affects both Cdc48 and ER homeostasis but plays no role in ERAD. Accordingly, we next tested the effect of Dfm1p overexpression on a mutant

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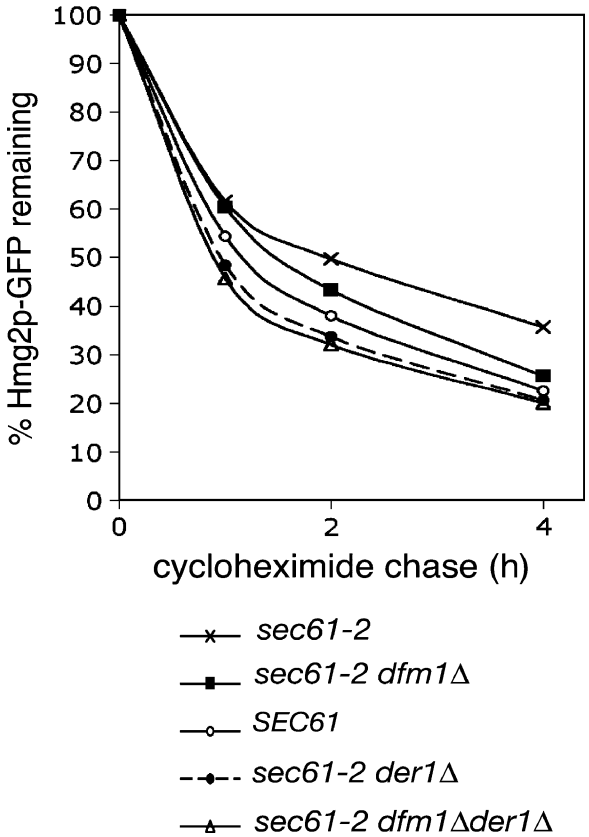

Figure 4. Null mutations of *DFM1* or *DER1* did not exacerbate the ERAD defect of a *sec61* mutant. The indicated strains expressing Hmg2p-GFP were grown into log phase and degradation was measured by a cycloheximide chase. CHX was added to isogenic cultures incubated at 30 °C at the indicated times to allow for simultaneous analysis of all cultures by flow cytometry at the end of the experiment

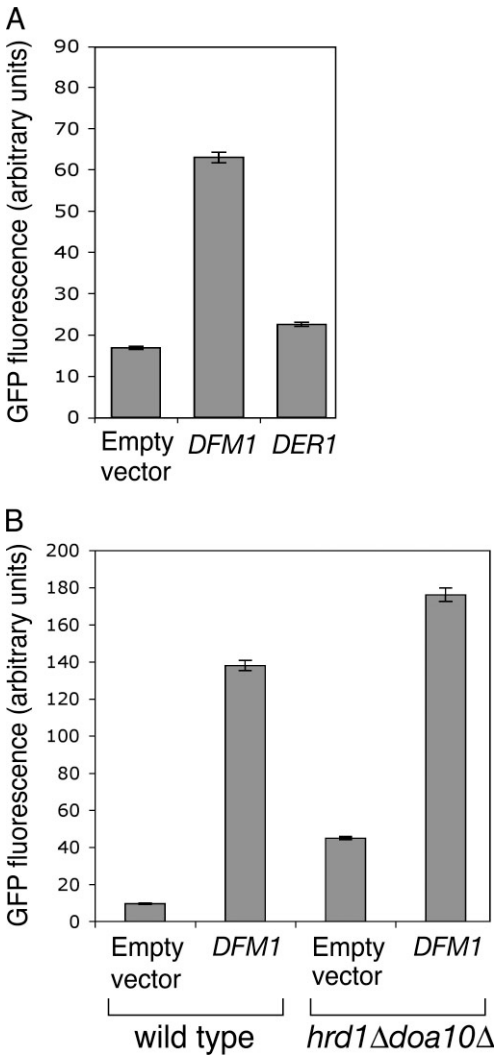

Figure 5. The unfolded protein response was upregulated by *DFM1* overexpression. (A). A wild type strain expressing the UPRE4-GFP reporter was transformed with empty vector or plasmids expressing *DFM1* or *DER1* driven by the strong TDH3 promoter. Cells were grown into log phase and then analyzed by flow cytemetry for UPR levels. (B). *DFM1*-stimulated UPR is not dependent on a functional ERAD pathway. Wild type and *hrd1Δdoa10Δ* strains with the UPRE-GFP reporter and harboring either empty vector or a *DFM1* overexpressing plasmid were compared for UPR

related to a non-ERAD function of Cdc48p, ER homotypic membrane fusion. This process requires the t-SNARE Ufe1p as well as Cdc48p (Latterich *et al*., 1995; Patel *et al*., 1998). We tested whether overexpression of Dfm1p produces the same phenotype in a temperature-sensitive *ufe1* mutant that we observed in the *cdc48* mutant. As was the case for the *cdc48* mutant, we observed a growth defect in the *ufe1-1* strain overexpressing Dfm1p (Figure 6C). This too was specific for Dfm1p, as overexpression of Der1p had no effect on growth. Thus, in addition to a genetic interaction with *CDC48, DFM1* interacts genetically with *UFE1*.

We further studied the relationship between the Dfm1p and Cdc48p phenotypes through mapping of the sequence determinants for this phenotype. The Dfm1p killing of *cdc48* is specific for that paralogue; the Der1p protein did not cause *cdc48* lethality. The most salient difference between the two paralogues is the extended C-terminal tail on Dfm1p (Figure 1). This portion of the protein is particularly interesting, since it contains two SHP

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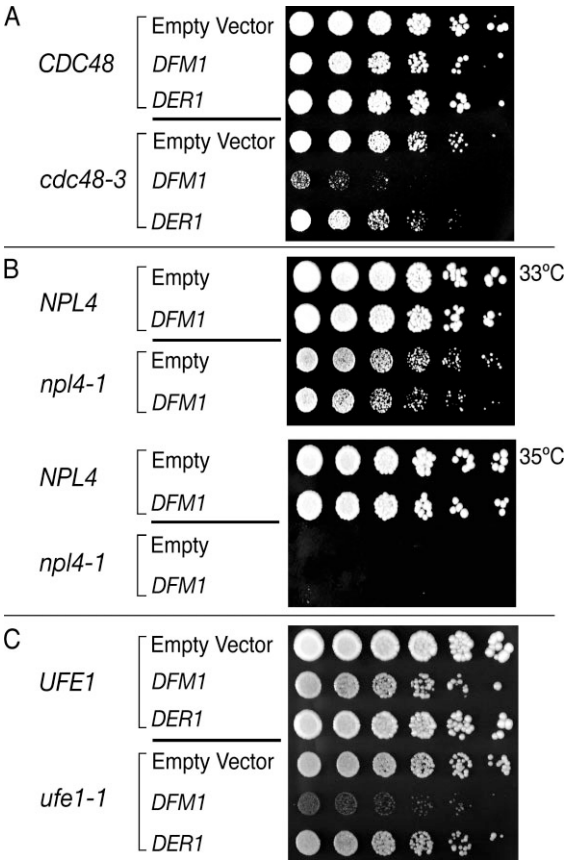

Figure 6. Genetic effects between *DFM1* and *CDC48* or Cdc48 Interaction partners. (A). Wild type and *cdc48-3* strains with empty vector or plasmids overexpressing *DFM1* or *DER1* were compared for growth by dilution assay. Each strain was spotted at 5-fold dilutions on solid media, and plates were incubated at 32 °C. (B). Lack of an effect of *DFM1* overexpression on an *npl4* mutant. A temperature sensitive *npl4-1* mutant was similarly tested for sensitivity to *DFM1* overexpression. Strains were grown as above and plates were incubated at 33 °C to show a lack of an effect of *DFM1* overexpression. An identical plate was incubated at 35 °C to demonstrate the ts- phenotype of the *npl4-1*. (C) Overexpression of *DFM1* inhibits the growth of an *ufe1-1* mutant. A temperature sensitive *ufe1-1* mutant was similarly tested for sensitivity to *DFM1* overexpression. Strains were grown as above and plates were incubated at 33 °C

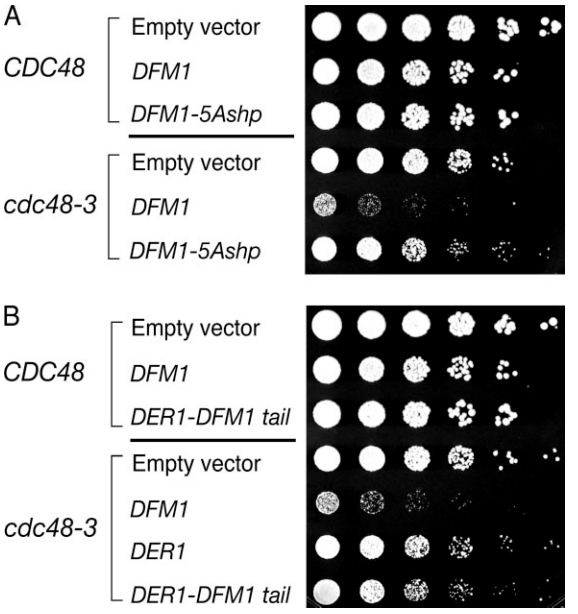

Figure 7. The *DFM1* C-terminal tail is necessary but not sufficient for the *cdc48-3* killing phenotype. (A-B) Wild type and *cdc48-3* strains expressing either empty vector, or plasmids overexpressing *DFM1*, *DER1*, *DER1-DFM1 tail*, or *DFM1-5Ashp* as indicated, were tested by dilution assay as above. Plates were incubated at 32 °C

box sequences, found in the SEP domain of Shp1p. The SEP domain is also a feature of mammalian p47 and the *Drosophila eyes closed* gene, and has been demonstrated to act as an interaction site between p97 and p47 (Sang and Ready, 2002; Yuan *et al*., 2004). We have analysed the role of the Dfm1p C-terminal tail and the significance of the SHP box in the genetic interaction of *CDC48* with *DFM1*.

We tested the importance of unique, C-terminal regions of Dfm1p, by making two *DFM1* mutants. We first mutated regions encoding the two SHP boxes of *DFM1*. The conserved sequence of F*x*G*x*GQR*n* (where *x* is a non-conserved amino acid and *n* is a non-polar amino acid) is present in Dfm1p at amino acids 284 (FSGRGQRL) and 324 (FQGRGQRV). We eliminated the SHP boxes by mutating all the conserved amino acids to alanine. Overexpression of this *dfm1* mutant lacking the SHP boxes ('*DFM1–5Ashp*') had no effect on *cdc48* lethality, demonstrating that these sequences are necessary for the phenotype (Figure 7A). This lends credence to the idea that these motifs mediate an interaction with Cdc48p. This effect was not due to differences in protein expression between wild-type and *cdc48* mutant strains, as both Dfm1p and *DFM1-5Ashp* had similar levels in each strain (DNS). Removal of the SHP box also blocked Dfm1p's lethal effect on *ufe1* mutants (DNS). We then created a fusion gene that produces Der1p with the added Dfm1p C-terminal tail. Like wild-type Der1p overexpression, Der1p with the added

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Dfm1 C-terminus had no effect on the growth of a *cdc48-3* strain (Figure 7B). Thus, the SHP box sequences are necessary for the Dfm1p dependent *cdc48* killing, but the C-terminal tail alone is not sufficient to cause this effect.

The genetic interaction between Dfm1p and Cdc48p implies that the two proteins physically interact in a SHP box-dependent manner. We tested for a direct interaction with a co-immunoprecipitation experiment. We prepared strains expressing 3HA-Dfm1p alone or with a functional protA-Cdc48p fusion. The tagged Dfm1p construct caused the same overexpression phenotypes observed with untagged Dfm1p (DNS). Detergent lysates were prepared, and the protA-Cdc48p was precipitated from the lysates with IgG-coupled beads. The lysates were then immunoblotted with anti-Cdc48 antibodies or anti-HA to detect co-precipitated 3HA-Dfm1p. The Dfm1p was co-precipitated by the IgG beads, but only in strains that also expressed the protA-Cdc48p fusion (Figure 8). As expected from the overexpression studies above, the SHP boxes were required for co-precipitation of Dfm1p by Cdc48p. Dfm1p with mutant SHP boxes did not interact with Cdc48p, as indicated by the absence of any additional HA signal above background when protA-Cdc48p is present in the precipitation. Interestingly, the *dfm1* mutant missing the SHP boxes is somewhat more abundant than the wild-type, so the loads were adjusted after precipitation to allow facile comparison with the strains expressing wild-type 3HA-Dfm1p.

Overexpressing Dfm1p had two phenotypes: UPR stimulation and killing of *cdc48* strains. We next evaluated the relationship between these two effects, using the Dfm1p variants generated above. It has been shown that *cdc48* mutants have an elevated UPR (Ye *et al*., 2001). Since Dfm1p and Cdc48p interact, one possibility was that Dfm1p overexpression caused sequestration of Cdc48p, thus elevating the UPR. This was not the case, as the two Dfm1p overexpression phenotypes showed entirely distinct sequence requirements. As shown above, elimination of the Dfm1 SHP boxes removed the *cdc48* killing phenotype. Conversely, overexpression of this *5Ashp* mutant still caused robust UPR that was, in fact, slightly higher caused by the wild-type Dfm1p (Figure 9). Furthermore, overexpression of a Der1p fusion with the C-terminal tail of Dfm1p, which had no growth effect

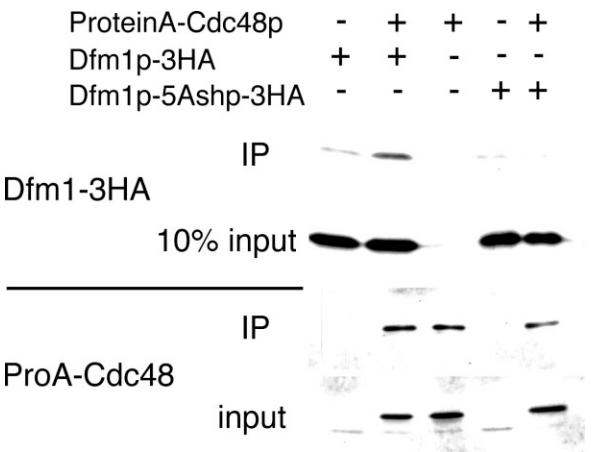

Figure 8. Dfm1p binds Cdc48p in a SHP box dependent manner. Strains expressing the indicated proteins were grown and equal amounts were harvested. Detergent lysate of ER-enriched microsomes were prepared as described and precipitated with IgG-beads to pull-down protein A-Cdc48. The precipitates were analyzed by SDS-PAGE and immunoblotted for either anti-HA monoclonal antibody or anti-CDC48 polyclonal antibody

on *cdc48-3* (Figure 7B), caused strongly upregulated UPR, similar to wild-type Dfm1p (Figure 9). It appears, in the context of a Der1p fusion, that the Dfm1p C-terminal tail is sufficient to cause the UPR phenotype, whereas it is not sufficient to cause *cdc48* killing. Thus, the UPR effect and *cdc48* lethality have separable sequence requirements, indicating that the UPR upregulation is not due to Dfm1p sequestration of Cdc48p.

Discussion

The mammalian Derlins have generated great interest due to their observed role in ER degradation. Their participation in the p97-dependent part of the virally-mediated MHC-I degradation pathway implies they may have a general role in the retrotranslocation phase of ER degradation. Because many aspects of ERAD are conserved between yeast and mammals, we explored the function of the yeast Derlin homologue *DFM1*, focusing on its possible functions in both ER homeostasis and as an interaction partner for the yeast p97 homologue Cdc48.

Recent studies suggested that Dfm1p is not involved in ER degradation by direct analysis of several substrates or phenotypes (Hitt and Wolf,

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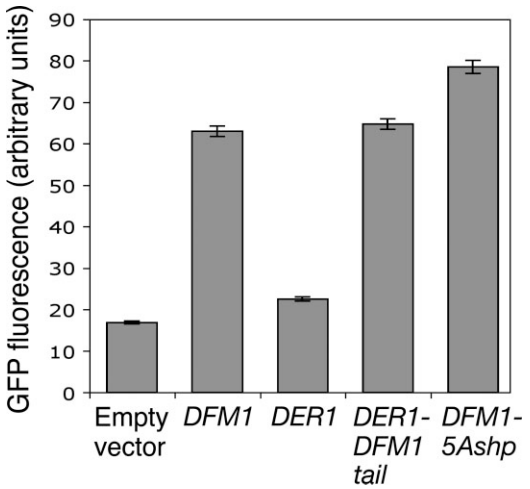

Figure 9. Sequence determinants of UPR induction by *DFM1*: the Dfm1 tail is sufficient for UPR induction. A strain expressing the UPRE4-GFP reporter was transformed with the indicated overexpression constructs. Strains were grown into log phase and GFP fluorescence was measured by flow cytometry

2004; Kreft *et al*., 2006). Consistent with these earlier studies, *DFM1* was not required for the degradation of a number of Der1-dependent and independent substrates. Furthermore, Dfm1p was not redundant with Der1p or Sec61p. However, we found that altering the levels of Dfm1p clearly affected ER stress. Loss of *DFM1* exacerbated the UPR in a *der1Δ* null mutant, while overexpression caused significant UPR in a wild-type strain. The overexpression phenotype was surprising, since in our studies we have never observed this effect upon overexpression of numerous membrane proteins, such as ERAD substrates. In addition, we demonstrated both genetic and physical interaction between Dfm1p and Cdc48p, which was dependent on a novel Cdc48-binding motif found in the C-terminus of Dfm1p. Finally, the UPR and *cdc48* interaction functions could be cleanly separated, indicating that Dfm1p plays a multifaceted role in non-ERAD, ER-related functions of the Cdc48p protein.

The Dfm1 UPR result is comparable in intensity to the response caused by overexpression of CPY*, a misfolded ERAD substrate (Knop *et al*., 1996). However, we speculate that CPY* and Dfm1p overexpression may cause UPR by a different mechanism. CPY* is a misfolded protein, and its strong expression causes sustained ER stress due to its detection as a client protein (Knop *et al*., 1996). In contrast, we suspect Dfm1p alters UPR by engaging molecules from the signalling pathway itself, rather than by being an abundant, misfolded ER protein.

Dfm1p displayed highly specific genetic and physical interactions with Cdc48p. Overexpression of Dfm1p caused a drastic decrease in the viability of *cdc48* mutant strains and this effect required the SHP boxes. Consistent with this action, Dfm1p directly binds Cdc48p in co-immunoprecipitation assays in a SHP box-dependent manner. These studies define the SHP box as a distinct Cdc48p interaction domain. This eight-amino acid sequence is found in other *S. cerevisiae* proteins as well, including Shp1p, Ufd1p and Rpn1p. Shp1p and Ufd1p are known Cdc48 binding proteins and Rpn1p is a proteasome subunit that could potentially interact with the Cdc48 complex. Studies performed by Ye *et al*. (2003) have identified the C-terminus of mammalian Ufd1 as a requirement for p97 binding. Not surprisingly, the SHP box in Ufd1p is located in the C-terminus as well. It will be interesting to learn whether interactions between Cdc48p and other SHP box containing proteins are similarly dependent on this motif.

Dfm1p does not appear to play a role in ERAD, despite our and others' extensive efforts to discover such a function. It is still possible that it has an ancillary ERAD function that we have yet to discover. Perhaps Dfm1p is not usually required for ERAD in normal conditions, but becomes limiting under abnormal circumstances, such as stressful growth conditions. However, several hours of heat stress applied to *dfm1Δ* cells did not alter the degradation kinetics of CPY* or Hmg2p (DNS). Another possibility is that it is a negative regulator of ERAD, in which case the null condition would not result in inhibition of substrate degradation. However, the *dfm1Δ* null mutant did not hasten degradation of any of the ERAD substrate studied above.

Dfm1p's interaction with Cdc48p implies that it participates in one of the several other functions of Cdc48p. Originally, *CDC48* was isolated in a screen for mutants that inhibit the cell cycle in yeast (Moir *et al*., 1982). It has been speculated that this cell cycle block is due to a role for Cdc48p in nuclear membrane fusion. In addition, both Cdc48p and p97 have been implicated in disassembly of mitotic spindles after anaphase (Cao *et al*., 2003).

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While Cdc48p has strong functional ties to cell cycle regulation, it is unlikely that Dfm1p has a positive action in these functions, since they are essential and loss of Dfm1p is not lethal. Alternatively, Dfm1p may play a modulatory role in one of these essential functions, and in this way exacerbate the phenotypes of *cdc48* hypomorphs upon overexpression.

Another function of Cdc48p is the mediation of ER homotypic fusion. Latterich *et al*. (1995) demonstrated that Cdc48p is required for ER homotypic membrane fusion. This process also requires Ufe1p, an ER localized t-SNARE. The current model is that Ufe1p binds to another Ufe1p molecule, resulting in fusion; this Ufe1p complex is then dismantled through the actions of Cdc48p (Patel *et al*., 1998). The authors demonstrated that an *ufe1-1* mutant is severely inhibited for ER homotypic fusion at 30 °C, despite a lack of obvious growth defects. Although *ufe1Δ* strains are non-viable due to the many roles Ufe1p plays, the possibility that ER homotypic membrane fusion is not essential makes it an appealing candidate for Dfm1p's function. Consistent with this idea, we observed that overexpression of Dfm1p inhibited the growth of an *ufe1-1* mutant in a SHP-box dependent manner. Furthermore, killing of *ufe1* mutants was also dependent on the SHP boxes of Dfm1p. Conversely, Dfm1p overexpression did not affect temperature-sensitive *npl4* mutants, the protein that associates with Cdc48p and functions in ERAD. Thus, it appears that Dfm1p functions at the ER in conjunction with Cdc48p in a process distinct from ERAD, with the best candidate being homotypic fusion of the ER. We are currently testing the role of Dfm1p in this and other functions of Cdc48p.

How does the function of Dfm1p connect to the mammalian Derlins? Both form complexes with p97 homologues, and appear to function with these proteins. One possibility is that the two proteins are sufficiently diverged that their functional interactions with p97/Cdc48p are distinct; so that the yeast form is not involved in ERAD, while the mammalian form is. Alternatively, it may be that in both organisms, Derlins function in non-ERAD dependent functions of p97/Cdc48p, and mammalian HCMV hijacks the p97-associated Derlin complex to employ it as a novel route of p97-mediated ERAD. There are other examples of virally-mediated rerouting of functions for targeted protein degradation. For example, human immunodeficiency virus type-1 (HIV) produces a protein, Vpu, that is involved in ERAD of CD4 (Meusser and Sommer, 2004). To effect this, Vpu recruits a cytoplasmic E3 ubiquitin ligase, β-TrCP that normally has no role in ERAD (Meusser and Sommer, 2004). Similarly, the human papillomavirus E6 protein programmes degradation of p53 by recruiting the E6AP E3 ligase which does not normally target p53 (Scheffner *et al*., 1993).

In any case, it is clear that the interaction of Derlins with p97/Cdc48p is a long-standing one, and could well be involved in a variety of actions of this essential and widely used AAA ATPase.

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