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A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation

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We developed a growth test to screen for yeast mutants defective in endoplasmic reticulum (ER) quality control and associated protein degradation (ERAD) using the membrane protein CTL*, a chimeric derivative of the classical ER degradation substrate CPY*. In a genomic screen of ~5,000 viable yeast deletion mutants, we identified genes necessary for ER quality control and degradation. Among the new gene products, we identified Dsk2p and Rad23p. We show that these two proteins are probably delivery factors for ubiquitinated ER substrates to the proteasome, following their removal from the membrane via the Cdc48–Ufd1–Npl4p complex. In contrast to the ERAD substrate CTG*, proteasomal degradation of a cytosolic CPY*–GFP fusion is not dependent on Dsk2p and Rad23p, indicating pathway specificity for both proteins. We propose that, in certain degradation pathways, Dsk2p, Rad23p and the trimeric Cdc48 complex function together in the delivery of ubiquitinated proteins to the proteasome, avoiding malfolded protein aggregates in the cytoplasm.

Keywords: ER-associated degradation; proteasome; ubiquitin; Dsk2p; Rad23p

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INTRODUCTION

The endoplasmic reticulum (ER) is the site of entry for the secretory pathway, which supplies the ER, Golgi apparatus, plasma membrane, lysosome (vacuole) and cellular exterior with the protein equipment necessary for proper cellular function. Once in the ER, proteins are folded and modified to acquire their native, functional conformations. Failure of this process results in inactive proteins forming insoluble aggregates, which ultimately lead to cellular malfunction and unhealthy cellular states (Kopito, 2000; Kostova & Wolf, 2003). The ER contains an efficient protein quality control system that recognizes malfolded and orphan proteins (Ellgaard *et al*, 1999) and targets them for elimination through a process known as ER-associated protein degradation (ERAD), to ensure delivery only of properly folded proteins to their site of action. Elimination is achieved via retrograde transport back to the cytoplasm through a channel possibly containing the Sec61p protein (Kostova & Wolf, 2003). While still associated with the ER membrane, proteins are polyubiquitinated. Thereafter, an AAA–ATPase complex composed of Cdc48p (p97 in mammalian cells), Ufd1p and Npl4p binds the polyubiquitinated proteins and mediates their release from the membrane. The 26S proteasome finally degrades the ubiquitinated substrates. The proteasome consists of the 20S cylindrical core, which is responsible for hydrolysis of ubiquitinated proteins, and the 19S regulatory particle that has a number of functions, including substrate recognition, binding and unfolding, opening of the 20S core and driving of the polypeptide chain through this opening (Kostova & Wolf, 2003). The identity of proteins that recognize polyubiquitinated substrates and deliver them to the proteasome for degradation is a subject of long-standing discussion. Earlier findings had suggested that Rpn10 (S5a in mammalian cells), a 19S cap subunit containing ubiquitin-interacting motif, might be the subunit binding polyubiquitinated substrates. However, deletion of this subunit is not lethal in several organisms (van Nocker *et al*, 1996; Fu *et al*, 1998). Crosslinking experiments have indicated that the Rpt5 (S6′) ATPase subunit of the 19S cap complex interacts with polyubiquitin chains (Lam *et al*, 2002). Recently, two other polyubiquitin-binding proteins, Dsk2p and Rad23p, have been identified (Wilkinson *et al*, 2001; Chen & Madura, 2002; Funakoshi *et al*, 2002; Hartmann-Petersen *et al*, 2003). Dsk2p and Rad23p are not 19S cap subunits, but participate in spindle-pole body duplication (Biggins *et al*, 1996) and nucleotide excision repair (Watkins *et al*, 1993), respectively. Rad23p has also been proposed to bind N-glycanase, an enzyme involved in deglycosylating carbohydrate-containing substrates of the proteasome (Suzuki *et al*, 2001). Dsk2p and Rad23p possess an N-terminal ubiquitin-like domain (UBL), which binds to a specific site on the 19S cap, and a C-terminal ubiquitin-associated domain(s) (UBA), capable of binding polyubiquitin chains (Wilkinson *et al*, 2001; Rao & Sastry, 2002; Hartmann-Petersen *et al*, 2003). These characteristics suggest that degradation substrates can bind to the Dsk2p or Rad23p UBA domain through their polyubiquitin chain and, consequently, can be delivered to the proteasome by means of the UBL–19S cap interaction. However, there is controversy about assigning this role to Dsk2p and Rad23p. Although degradation of ubiquitin-fusion

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degradation (UFD) substrates depends on these two proteins (Chen & Madura, 2002; Rao & Sastry, 2002), in some *in vitro* studies an inhibitory effect of Rad23p on proteasome activity has been noted (Raasi & Pickart, 2003).

Here, we report a genome-wide screen carried out to identify new yeast mutants with defects in ER protein quality control and degradation. We identify Dsk2p and Rad23p as new components with overlapping functions involved in the ERAD of two topologically different but otherwise related substrates. On the basis of our data and previous findings, we propose that Dsk2p and Rad23p function as delivery factors between the Cdc48 complex and the proteasome, and that they are specific to certain ubiquitin–protein degradation pathways.

RESULTS AND DISCUSSION

To gain a deeper insight into the molecular mechanisms of protein quality control and ER-associated degradation, we undertook a genome-wide screen using the EUROSCARF yeast library, consisting of about 5,000 *Saccharomyces cerevisiae* strains, each deleted for a single non-essential gene. The use of this library for screening mutants of ERAD is possible because cells tolerate a defect in this process as long as the unfolded protein response is intact (Friedländer *et al*, 2000; Travers *et al*, 2000). Cell growth is one of the most sensitive indicators of alterations in cell physiology due to mutations. It is known that yeast cells expressing a mutated version of the Sec61p translocon, Sec61-2p, are not viable at elevated temperature (37 °C) because Sec61-2p becomes an ERAD substrate (Biederer *et al*, 1996). Viability under these conditions is restored only in the absence of key ERAD components, like Der3/Hrd1p, Ubc6p and Ubc7p (Biederer *et al*, 1996; Bordallo *et al*, 1998). On the basis of this growth phenotype of yeast strains defective in ERAD, we devised a screen to identify new ERAD components. We have previously described the membrane-localized ERAD substrate CTG*, which consists of an ER-lumenal malfolded CPY* domain connected to green fluorescent protein (GFP) in the cytoplasm via a transmembrane domain (Taxis *et al*, 2003). To screen for new mutants, we replaced the cytoplasmic GFP moiety with the Leu2 protein (3-isopropylmalate-dehydrogenase) (Fig 1A). This new construct, called CTL*, was placed under the control of the weak *GAL4* promoter (Griggs & Johnston, 1993). Low expression of CTL* was particularly required because yeast cells can sustain growth even in the presence of minimal amounts of leucine. Consequently, strains with *leu2* auxotrophy but, otherwise, wild type for ERAD are unable to grow in media lacking leucine (Fig 1B). Only when ERAD is defective is CTL* stabilized and able to complement the *leu2* deficiency (Fig 1B). The low expression then allows for sharp growth differences to be easily observed. We took advantage of this growth phenotype to screen the ~5,000 individual deletion mutants of the EUROSCARF yeast library expressing CTL*. Strains defective in most of the known ERAD components resulted in growth in the absence of leucine (Table 1), highlighting the reliability of the selection procedure. The screen yielded a number of new mutants exhibiting a reproducible growth phenotype. Among these, we observed strong complementation in strains carrying the *dsk2* and, to a lesser extent, *rad23* deletions (Fig 1B). To test whether these two proteins are true components of the ER degradation machinery, we analysed the degradation of two well-known ERAD substrates, soluble CPY* (Hiller *et al*, 1996) and membrane-anchored CTG* (Taxis *et al*, 2003), in a set of isogenic strains deleted in either one or both genes. Pulse-chase analyses showed a strong stabilization of CPY* haemagglutinin (HA) and CTG* in the Δ*dsk2*Δ*rad23* double mutant, indicating that these proteins participate directly in ERAD (Fig 2). CPY*HA and CTG* exhibited about 10% stabilization in Δ*dsk2* and Δ*rad23* single mutants, and 40% and 45% stabilization, respectively, in the Δ*dsk2*Δ*rad23* double mutant with respect to wild-type cells

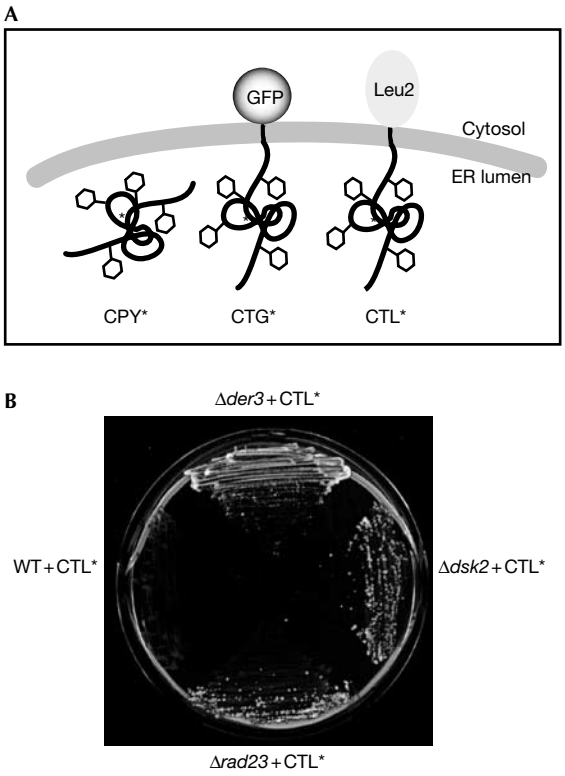

Fig 1 | *DSK2* and *RAD23* are found in a screen for new ERAD components. (**A**) Schematic representation of the ERAD substrates CPY*, CTG* and CTL*. (**B**) The BY4743 wild-type (WT) strain expressing CTL* fails to grow on SC medium lacking leucine, whereas a strain deleted in the well-known ERAD component Der3p, used as a positive control, permits growth. The deletion strains Δ*dsk2* and Δ*rad23*, expressing CTL*, complement the leucine auxotrophy of the strain.

Table 1 | Known ERAD components found in the screen

ORF name	Gene name	Function
YOL013C	*HRD1/DER3*	Ubiquitin-protein ligase
YLR207W	*HRD3*	Der3p interacting protein
YHR204W	*HTM1/MNL1*	ER-lumenal lectin
YNR030W	*ECM39/ALG12*	N-glycan synthesis
YJR131W	*MNS1*	ER α-mannosidase I
YMR022W	*UBC7*	Ubiquitin-conjugating enzyme
YEL031W	*SPF1/COD1*	ER calcium pump

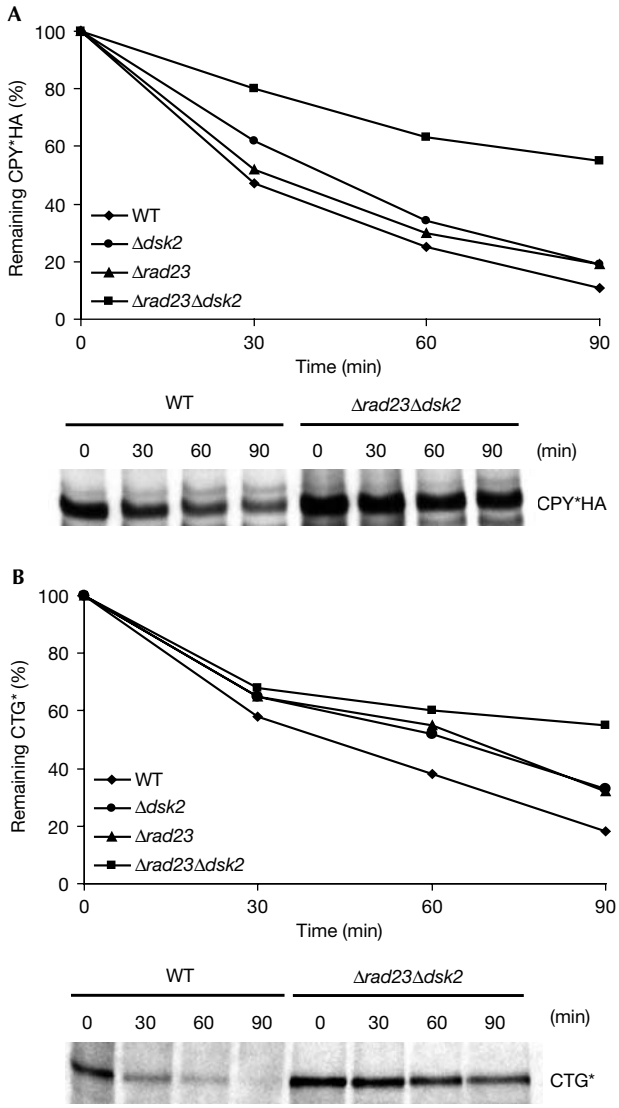

Fig 2 | Mutants deleted in *DSK2* and *RAD23* are defective in the degradation of the ERAD substrates CPY*HA and CTG*. (**A**,**B**) Yeast strains expressing CPY*HA or CTG* were metabolically labelled, chased for the indicated times and immunoprecipitated using anti-HA or anti-CPY antibodies, respectively. Δ*dsk2* and Δ*rad23* single- and double-deletion mutants show synergistic effects in ERAD. Degradation of CPY*HA (**A**) and CTG* (**B**) is significantly delayed in a Δ*dsk2*Δ*rad23* strain. Data represent the mean values of three (CPY*HA) and two (CTG*) independent experiments. WT, wild type.

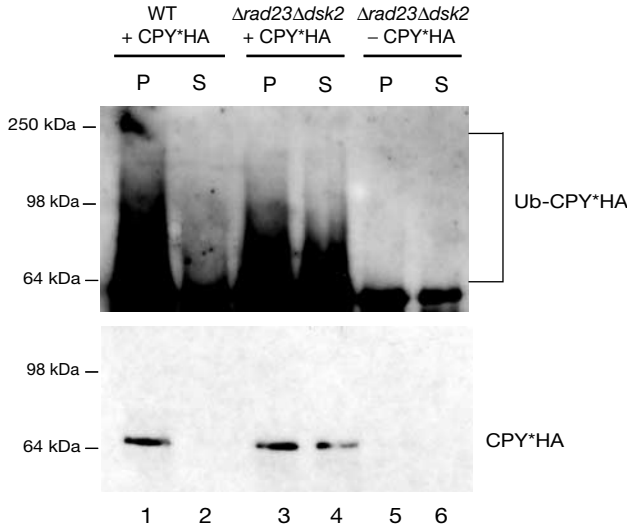

Fig 3 | Polyubiquitinated CPY*HA accumulates in the cytosol of the Δ*dsk2*Δ*rad23* mutant. Membrane association of ubiquitinated CPY*HA was investigated in respectively transformed wild type (WT) and Δ*dsk2*Δ*rad23* strains. Microsomal extracts were separated into membrane-associated (P) and cytosolic (S) fractions. CPY*HA was precipitated with anti-CPY antibodies and analysed by immunoblotting with anti-ubiquitin antibodies. The pellet and supernatant fractions from non-transformed strain Δ*dsk2*Δ*rad23* are included to reveal the background activity of the ubiquitin antibody. Immunoblotting with HA-antibody shows localization of CPY*HA in each fraction.

(Fig 2A,B). This synergistic effect implies that Dsk2p and Rad23p function together in ERAD, consistent with reports implicating Dsk2p and Rad23p in the proteasomal turnover of the artificial substrates Ub^{V76}-V-β-gal and Ub-P-β-gal (Chen & Madura, 2002; Rao & Sastry, 2002) via the UFD pathway. Fractionation of microsomes derived from wild-type and *ufd1-1* (a mutant allele of Ufd1p, a component of the AAA–ATPase Cdc48p–Ufd1p–Npl4p complex (Ye *et al*, 2001; Jarosch *et al*, 2002)) strains results in a considerable portion of ubiquitinated CPY* to be retained in the pellet, with only a minor amount present in the soluble fraction. In wild-type cells, the ubiquitinated material released by the trimeric Cdc48 complex into the supernatant is rapidly degraded by proteasomes. In *ufd1-1* cells there is no cytoplasmic ubiquitinated protein product owing to a defective trimeric Cdc48 complex (Jarosch *et al*, 2002). To elucidate the step at which Dsk2p and Rad23p may be involved, we examined the localization of ubiquitinated material in wild-type and Δ*dsk2*Δ*rad23* strains. As shown previously, we found little ubiquitinated CPY*HA in the soluble fraction of wild-type cells compared with the pellet (Fig 3, lanes 1 and 2). As expected, there was no CPY*HA visible in the supernatant owing to degradation via the proteasome. In comparison, a considerably larger amount of ubiquitinated protein was detected in the soluble fraction of the Δ*dsk2*Δ*rad23* double mutant (Fig 3, lane 4; compare lanes 4 and 2). These findings indicate that ubiquitinated CPY*HA accumulates in the cytosol as a consequence of failed delivery to the proteasome, and are in agreement with a role for Dsk2p and Rad23p downstream of the Cdc48–Ufd1–Npl4 complex, targeting substrates for proteasomal degradation.

We also investigated whether Dsk2p and Rad23p are general components of the targeting/degradation machinery of ubiquitinated proteins. We constructed ΔssCPY*-GFP, which lacks the CPY signal sequence, as a representative soluble proteasomal substrate. Cell fractionation, protease protection and deglycosylation experiments verified that ΔssCPY*-GFP, which carries the same mutated motif as the ER-localized CPY* and CTG*, resides in the cytoplasm (Fig 4). Next, we determined whether cytoplasmic ΔssCPY*-GFP is a substrate of the 26S proteasome.

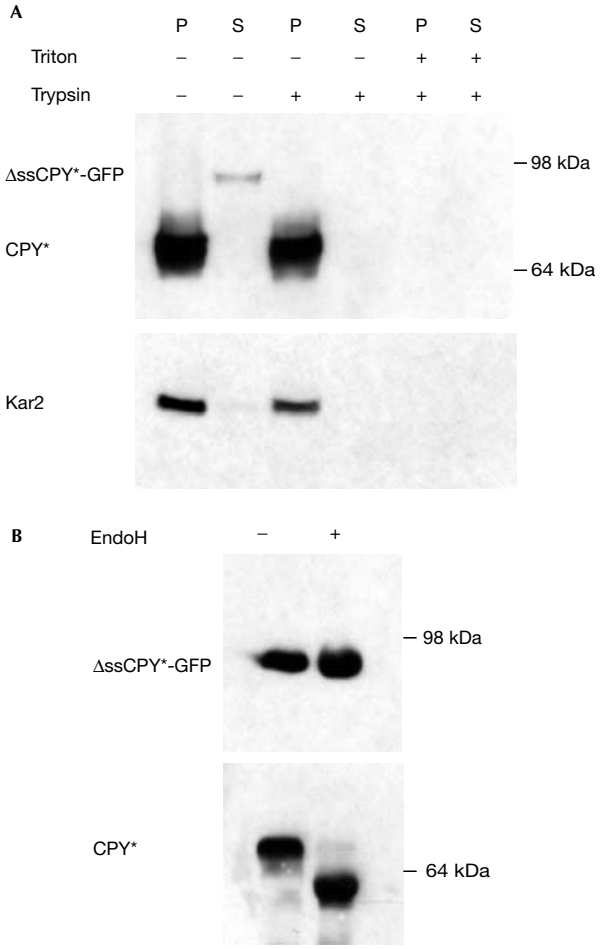

Fig 4 | ΔssCPY*-GFP is located in the cytosol. (**A**) Spheroplasts from strain YCT397 transformed with ΔssCPY*-GFP and chromosomally expressing CPY* were isolated and fractionated into microsome pellet (P) and supernatant (S). Both fractions were treated with trypsin and triton as indicated. The ER lumenal Kar2p was used as a control for fractionation and protease protection accuracy. (**B**) ΔssCPY*-GFP is not glycosylated. ΔssCPY*-GFP and, as a control, glycosylated CPY* were immunoprecipitated using CPY antibodies from YCT397 expressing both proteins (see (**A**)), subjected to endoglycosidase H (EndoH) treatment and immunoblotted. ΔssCPY*-GFP was detected with anti-GFP; CPY* was detected with anti-CPY antibodies.

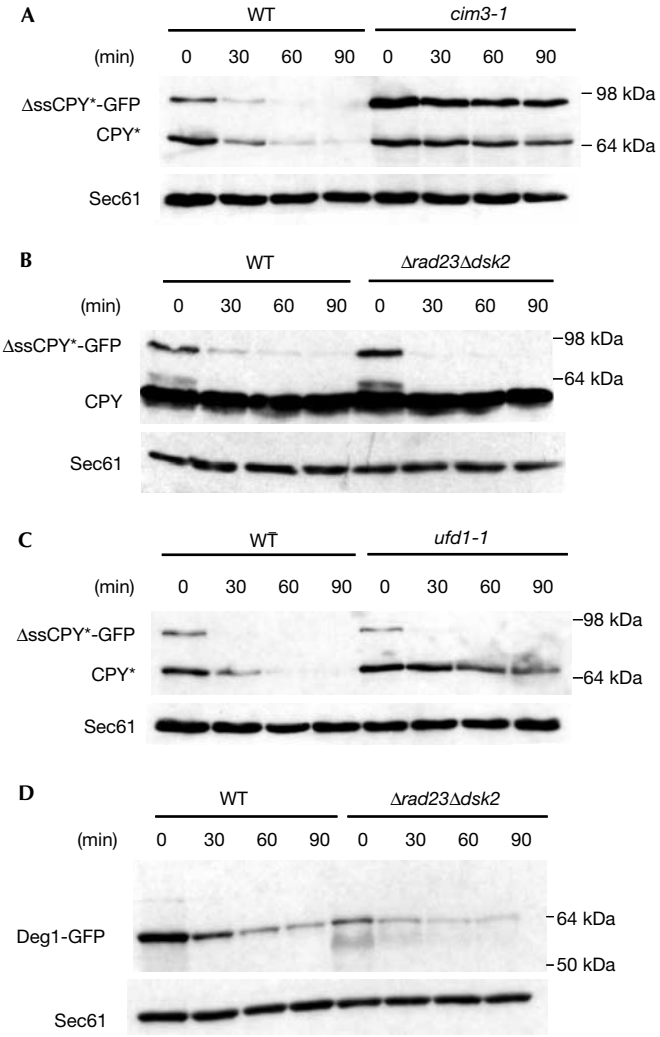

Fig 5 | Degradation of cytosolic CPY*-GFP and Deg1-GFP is independent of Dsk2p and Rad23p. (**A**) Cycloheximide decay experiments performed in wild type (WT) and proteasomal mutant *cim3-1* strains expressing both CPY* and ΔssCPY*-GFP show that ΔssCPY*-GFP is a substrate of the 26S proteasome. (**B**) Cycloheximide decay experiments performed in WT and Δ*dsk2*Δ*rad23* strains expressing CPY and ΔssCPY*-GFP indicate that Dsk2p and Rad23p have no role in the degradation of cytosolic ΔssCPY*-GFP. (**C**) Degradation of ΔssCPY*-GFP is independent of Ufd1p. ER lumenal CPY*, conversely, is stabilized in the *ufd1-1* mutant. (**D**) Degradation of Deg1-GFP is independent of Dsk2p and Rad23p. Sec61p was used as a control for protein loading.

We found that ΔssCPY*-GFP is rapidly degraded in wild-type cells, whereas degradation is abolished in a mutant (*cim3-1*) defective in one of the 19S cap AAA–ATPase subunits (Rpt6p) (Hiller *et al*, 1996) (Fig 5A). Cycloheximide chase analyses revealed that degradation of cytoplasmic ΔssCPY*-GFP is not affected by the absence of Dsk2p and Rad23p: the protein is degraded as rapidly in the Δ*dsk2*Δ*rad23* double mutant as it is in wild-type cells (Fig 5B), underscoring the pathway-specific requirement for Dsk2p and Rad23p. Interestingly, both the ERAD and UFD pathways, which require Dsk2p and Rad23p function, are also dependent on the Cdc48–Ufd1–Npl4p complex (Ye *et al*, 2001; Jarosch *et al*, 2002). In fact, Ufd1p, the central subunit of the trimeric Cdc48 complex, was first identified as a component of the UFD pathway (Johnson *et al*, 1995). Therefore, we tested whether Ufd1p is necessary for the degradation of cytoplasmic ΔssCPY*-GFP. Consistent with the fact that Dsk2p and Rad23p are not involved, we observed no difference in the degradation of ΔssCPY*-GFP in wild-type or *ufd1-1* cells (Fig 5C). As a second, mainly cytoplasmic, substrate of the ubiquitin–proteasome system, we tested a fusion protein consisting of the Deg1 degradation domain of the MATα2 repressor protein and GFP, Deg1-GFP (Lenk & Sommer, 2000). The half-life of Deg1-GFP was determined by

pulse-chase analysis in wild-type and *ufd1-1* mutant cells as 22.5 and 24.5 min, respectively (data not shown), in agreement with published results (Lenk & Sommer, 2000). Cycloheximide chase experiments showed that Deg1-GFP is also not a target of Dsk2p and Rad23p (Fig 5D). On the basis of these findings, we propose that degradation pathways that require the AAA–ATPase complex Cdc48–Ufd1–Npl4p may also depend on Dsk2p and Rad23p, a concept that will require further exploration. In this scenario, substrates liberated from the trimeric Cdc48 complex are passed to Dsk2p and/or Rad23p, which, in turn, transfer them to the proteasome for degradation. We believe that, in this way, malfolded substrates of the ER are handed over to the proteasome following an uninterrupted path from the ER membrane, thus preventing the formation of insoluble protein aggregates in the cytoplasm.

We also propose that, contrary to previous interpretations that Dsk2p and Rad23p are essential for proteasomal targeting of ubiquitinated substrates in general or that they act as inhibitors in these same processes, the action of Dsk2p and Rad23p is not all-or-none, but pathway specific and finely tuned.

METHODS

Yeast strains and bacterial constructs. Molecular biological and genetic techniques were carried out using standard methods (Guthrie & Fink, 1991). *Saccharomyces cerevisiae* strains MY3589 (*MATa, ura3-52, leu, ade2*), MY3588 (*MATa, ura3-52, trp1, ade2, his3, dsk2*Δ::*LEU2*) and MY3592 (*MAT*α*, ura3-52, leu2, his3*Δ*200, ade2, dsk2*Δ::*LEU2, rad23*Δ) were described in Biggins *et al* (1996). MY3587F (*MAT*α*, ura3-52, leu2, his3, ade2, rad23*Δ) was described in Rao & Sastry (2002). Strains BY4743 (*MATa/*α*, his3*Δ*1/his3*Δ*1, leu2*Δ*0/leu2*Δ*0, lys2*Δ*0/LYS2, MET15/met15*Δ*0, ura3*Δ*0/ura3*Δ*0*), BY4743Δ*dsk2* (*MATa/*α*, his3*Δ*1/his3*Δ*1, leu2*Δ*0/leu2*Δ*0, lys2*Δ*0/LYS2, MET15/met15*Δ*0, ura3*Δ*0/ura3*Δ*0, dsk2*Δ::*kanMX4/ dsk2*Δ::*kanMX4*), BY4743Δ*rad23* (*MATa/*α*, his3*Δ*1/his3*Δ*1, leu2*Δ*0/leu2*Δ*0, lys2*Δ*0/LYS2, MET15/met15*Δ*0, ura3*Δ*0/ura3*Δ*0, rad23*Δ::*kanMX4/rad23*Δ::*kanMX4*) and BY4743Δ*der3/hrd1* (*MATa/*α*, his3*Δ*1/his3*Δ*1, leu2*Δ*0/leu2*Δ*0, lys2*Δ*0/LYS2, MET15/met15*Δ*0, ura3*Δ*0/ura3*Δ*0, der3*::*kanMX4/ der3*::*kan MX4*) are from the EUROSCARF Gene Deletion Bank (Frankfurt, Germany). YPH499Y (*MATa, ura3-52, leu2*Δ*1, his3*Δ*200, trp1*Δ*63, lys2-801, ade2-101, prc1-1*) and CMY762Y (*MATa, ura3-52, leu2*Δ*1, his3*Δ*200, cim3-1, prc1-1*) were previously described in Hiller *et al* (1996), whereas YCT397 (*MATa, ura3-52, leu2-3,112, his4-519, ade1-100, prc1-1*) and YCT415 (YCT397, *ufd1-1*) were described in Taxis *et al* (2003). Cells were grown at 25 °C (*ts* strain *cim3-1*) or at 30 °C in synthetic complete media.

3HA-tagged CPY* and CTG* were expressed from plasmids pCT42 and pMA1, respectively (Taxis *et al*, 2002, 2003). Deg1-GFP was expressed from plasmid pUL28 (Lenk & Sommer, 2000). pRS316 expressing Gal4-CTL* was cloned in a stepwise process: the *LEU2* gene was PCR-amplified using the primers 5′lPac (GGATCCTTAATTAAGTCTGCCCCTAAGAAG) and 3′lSph (GTA CAGGCATGCATAAATGTATGTAGATTG), and the 1.1 kb fragment was cloned into pNEB193 between *Pac*I and *Sph*I restriction sites. The 2.5 kb CT* fragment obtained by digesting pMA1 with *Kpn*I and *Pac*I was cloned into the respective sites of pNEB193-*LEU2*, yielding pNEB193-CTL*. CTL* was then transferred into pRS316 as a 3.7 kb *Hin*dIII fragment, giving rise to pRS316-tdh3-CTL*. The *TDH3* promoter of CTL* was replaced by the *GAL4* promoter as follows: the *GAL4* promoter was PCR-amplified using the primers 5′GAL4CTLNotI (AAATATGCGGCCGAGGACCCT GACGGCGA) and 3′GAL4CTL (GTAAACTGGTGAATGCTTT CATCTTTCAGGCTTGCTTC), the latter being partially complementary both to *GAL4* and the 5′ end of *prc1-1*. A 500 bp N-terminal fragment of CPY* was PCR-amplified with the primer set 5′CTLGAL4 (GAAGCAAGCCTCCTGAAAGATGAAAGCATTCAC CAGTTTAC), having partial complementarity both to CPY* and the 3′ end of the *GAL4* promoter, and 3′CTLBsu36I (GAAGTTA TAGACATCCTTACC). These two PCR products were used as overlapping templates in a third PCR to generate a final *promGAL4*::*prc1-1* fusion fragment, which was cloned into *Not*I–*Bsu36I*-digested pRS316-tdh3-CTL*, generating pRS316-gal4-CTL*. Cytosolic CPY*-GFP (ΔssCPY*-GFP) was cloned in two steps. First, we cloned pRS316CPY*-GFP via homologous recombination in yeast (details available upon request). Then, we removed the signal sequence from pRS316CPY*-GFP by a QuikChange (Stratagene) based PCR-mutagenesis approach, using the complementary primer set 5′PRC1(−)ss (GTATACATACGC TATGATCTCATTGCAAAGACCG) and 3′PRC1(−)ss (CGGTCTTT GCAATGAGATCATAGCGTATGTATAC).

Antibodies. Polyclonal anti-CPY was used for immunoprecipitation of CTG*. Monoclonal anti-CPY and monoclonal anti-GFP (Molecular Probes) and polyclonal anti-sec61 (gift from T. Sommer) were diluted 1:10,000 for immunoblotting. Monoclonal anti-HA (Covance) was used at 1:1,000 dilution for immunoprecipitation and at 1:10,000 dilution for immunoblotting. Monoclonal anti-ubiquitin antibody (Covance) was used at 1:1,000 dilution for immunoblotting.

Pulse-chase analysis and immunoprecipitation. CPY* and CTG* pulse-chase experiments were performed as described previously (Taxis *et al*, 2002), with the exception that cells were grown in 2% glucose and labelled for 60 min.

Cycloheximide decay analysis. Cells were grown at 30 °C to logarithmic phase in synthetic complete medium. The temperature-sensitive *cim3-1* strain was grown at 25 °C and then shifted to 30 °C for 1 h. Deg1-GFP, expressed under the control of the *CUP* promoter, was induced for 1 h by the addition of 100 μM $CuSO_4$. Cycloheximide (0.25 mg ml^{-1}) was added and 2 OD_{600} (6×10^7) of cells were taken at the indicated time points. Cell extracts were prepared by alkaline lysis and subjected to SDS–PAGE and western blotting as described in Taxis *et al* (2003).

Detection of ubiquitinated CPY*HA. Ubiquitinated CPY*HA was detected in pellet and supernatant fractions by following the procedure described in Hiller *et al* (1996).

Localization studies. For localization of ΔssCPY*-GFP protease protection (trypsin) and deglycosylation (endoglycosidase H), experiments were performed as described in Taxis *et al* (2002, 2003).

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