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Mobilization of Processed, Membrane-Tethered SPT23 Transcription Factor by CDC48$^{UFD1/NPL4}$, a Ubiquitin-Selective Chaperone

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Summary

The OLE pathway of yeast regulates the level of the ER-bound enzyme Δ9-fatty acid desaturase OLE1, thereby controlling membrane fluidity. A central component of this regulon is the transcription factor SPT23, a homolog of mammalian NF-κB. SPT23 is synthesized as an inactive, ER membrane-anchored precursor that is activated by regulated ubiquitin/proteasome-dependent processing (RUP). We now show that SPT23 dimerizes prior to processing and that the processed molecule, p90, retains its ubiquitin modification and initially remains tethered to its unprocessed, membrane-bound SPT23 partner. Subsequently, p90 is liberated from its partner for nuclear targeting by the activity of the chaperone-like CDC48$^{UFD1/NPL4}$ complex. Remarkably, this enzyme binds preferentially ubiquitinated substrates, suggesting that CDC48$^{UFD1/NPL4}$ is qualified to selectively remove ubiquitin conjugates from protein complexes.

Introduction

The endoplasmic reticulum (ER) is of fundamental importance for lipid and protein biosynthesis. It is the production site for lipids and sterols for the membranes of the ER, most of the cell's organelles, and the plasma membrane. Membrane composition is regulated at several levels. Sterol homeostasis is controlled at the ER by regulated degradation of the key enzyme HMG-CoA reductase (Hampton et al., 1996). Moreover, in mammalian cells, sterol biosynthesis is controlled by the transcription factor SREBP, which is synthesized as an inactive, ER membrane-bound precursor (reviewed by Brown et al., 2000). SREBP is liberated from the membrane by a sterol-controlled cleavage called regulated intramembrane proteolysis, or RIP, which involves two site-specific, membrane-bound proteases (reviewed by Brown et al., 2000).

Recently, a novel mechanism for the adjustment of unsaturated fatty acid (UFA) pools has been discovered in yeast cells (Hoppe et al., 2000). This pathway, termed the OLE pathway, controls the level of the ER-bound enzyme Δ9 fatty acid desaturase. The gene for this enzyme, *OLE1*, is activated by two transcription factors, SPT23 and MGA2 (Zhang et al., 1999). Both proteins are synthesized as inactive precursors (p120), which are anchored to the ER membrane via their single, carboxyl (C)-terminal transmembrane spans (Hoppe et al., 2000). Activation of SPT23 appears to be initiated by ubiquitination catalyzed by the RSP5 ubiquitin ligase, which interacts directly with SPT23 via one of RSP5's WW protein-protein interaction domains. Ubiquitination then promotes processing of the SPT23 p120 precursor, giving rise to a soluble shorter form (p90) that moves into the nucleus to drive *OLE1* transcription. High levels of UFAs completely block SPT23 processing, suggesting that SPT23 itself or interacting partners can sense the lipid composition of the membrane (Hoppe et al., 2000). SPT23 processing is not mediated by RIP, but rather by a reaction coined regulated ubiquitin/proteasome-dependent processing, or RUP, which involves ubiquitination and the 26S proteasome (Hoppe et al., 2000, 2001). RUP results in the complete degradation of the C-terminal, membrane-anchored domain of SPT23, whereas the amino (N)-terminal transcription factor domain is left intact. Processing of SPT23 by the proteasome was shown to occur virtually exclusively at the membrane (Hoppe et al., 2000). This suggests that processing of the membrane-bound SPT23 molecule is initiated by an internal cleavage event by which the polypeptide chain, through hairpin formation, reaches the active sites of the 26S proteasome, which are located in the central cavity of the protease. Intriguingly, SPT23 and MGA2 are distant homologs of the p105 precursor of NF-κB. Although p105 is a soluble protein, the mechanism by which the NF-κB precursor is processed is strikingly similar to SPT23 processing: it requires the ubiquitin/proteasome system and eliminates the C-terminal half of the protein, whereas the N-terminal transcription factor domain (p50) is kept intact (Lin et al., 2000 and references therein).

Here we show that SPT23 processing results in p90 bound to an unprocessed SPT23 p120 partner molecule at the membrane. Interestingly, p90 has retained its ubiquitin modification after processing, and we show that a chaperone-like enzyme, designated CDC48$^{UFD1/NPL4}$, separates p90 from p120 by an ATP-dependent mechanism, thereby mobilizing p90 for nuclear targeting. Intriguingly, the chaperone displays selectivity toward ubiquitinated proteins, suggesting that this enzyme has the potential to selectively remove ubiquitinated proteins from other proteins of oligomeric protein complexes.

Results

The IPT Domain of SPT23 Is Required for Dimerization and Processing

By doing a two-hybrid screen with SPT23, we found that SPT23 is able to homodimerize. The shortest isolate we obtained lacked the N-terminal half of SPT23 but retained the central IPT ("Ig-like/plexins/transcription factors") domain. Notably, we found that the smallest

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segment of SPT23 that is sufficient for homodimerization corresponds to the IPT domain, and that dimerization is mediated through IPT-IPT interaction (Figure 1A). Consistent with this finding, we observed that an SPT23 variant lacking the IPT domain (SPT23$^{\Delta IPT}$) failed to interact with the SPT23 bait in two-hybrid assays (Figure 1A). To investigate whether dimerization is important for SPT23 at the membrane, we expressed epitope-tagged SPT23 and also SPT23$^{\Delta IPT}$ in cells. Notably, the mutant protein, despite still being able to bind RSP5 in two-hybrid assays and being correctly inserted into the membrane, failed to be processed (Figure 1B and data not shown). This finding strongly suggests that dimerization is a prerequisite of SPT23 processing.

Processed SPT23 p90 Is Tethered to Membrane-Bound p120

SPT23 processing is essential to liberate the transcription factor from the membrane; indeed, the majority of processed p90 is detectable in the soluble fraction of cell free extracts (Hoppe et al., 2000). However, when we used buffers containing EDTA and protease inhibitors (PMSF) during cell fractionation to avoid proteolysis in vitro, a significant pool of p90 was observed in the microsomal pellet fraction (Figure 1C). This fraction of p90 was peripherally attached to membranes since it could be solubilized not only by detergents but also by treatment with carbonate buffers. Notably, p90 could not be washed off the membranes by sodium chloride at concentrations of up to 2.5 M (Figure 1D), indicative of a remarkably stable, possibly hydrophobic interaction.

To investigate whether the interaction of p90 with the membrane fraction is mediated by dimerization with unprocessed p120, we performed coimmunoprecipitation experiments. For that purpose, we used an SPT23 variant that contained an N-terminal myc- and a C-terminal HA-tag (^{myc}SPT23^{HA}; Hoppe et al., 2000). This construct allows us to distinguish between precursor and product forms of SPT23, because an HA-specific antibody exclusively detects p120, whereas p90 is only detected by anti-myc antibodies. As shown in Figure 1E, immunoprecipitation of SPT23 p120 solubilized by N-dodecyl-maltoside using the HA-antibody led to coprecipitation of significant amounts of p90. The two sets of data demonstrate that, following SPT23 processing, p90 remains tightly associated with a membrane-bound p120 partner molecule.

CDC48 and NPL4 Are Novel Components of the OLE Pathway

The above finding raised the question of how the transcription factor p90 is released from the membrane in vivo. A possible answer emerged upon the identification of additional components of the OLE pathway. Previously, we showed that UFD1 is required for the activation of SPT23/MGA2 transcription factors and hence for *OLE1* expression (Hoppe et al., 2000). Because UFD1 is also required for the degradation of unstable, artificial ubiquitin fusion proteins (the so-called UFD pathway; Johnson et al., 1995), we hypothesized that other components of the UFD pathway could possibly participate in the OLE pathway as well.

One particularly interesting candidate was the UFD

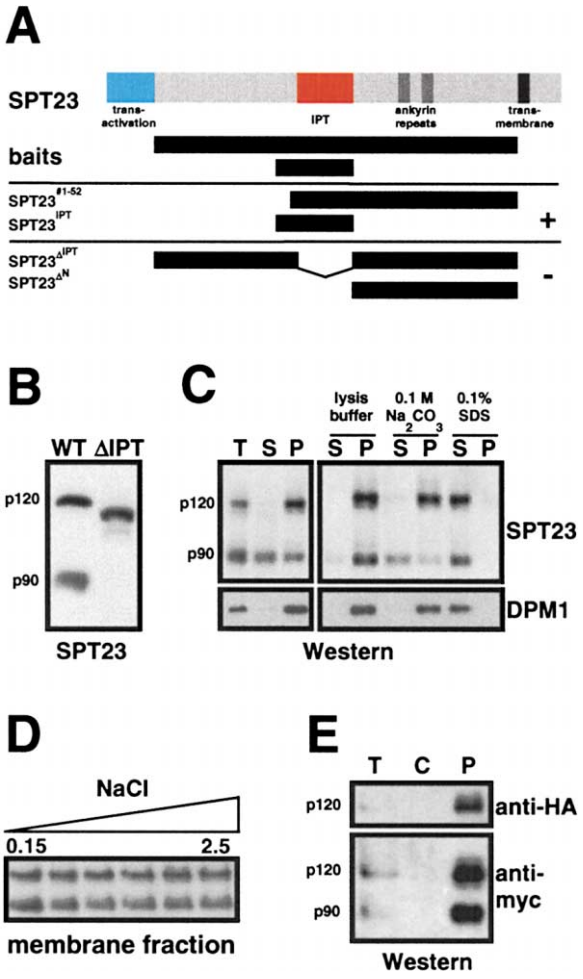

Figure 1. Association of SPT23 p90 with p120

(A) Yeast two-hybrid self-interaction of SPT23. Two different baits were used as schematically indicated by black bars. Interacting clones are designated with "+"; clones that do not interact with SPT23 are designated with "−". Note that the IPT domain is able to self-interact. SPT23$^{\#1-52}$ designates an isolate of a two-hybrid screen.

(B) Processing of SPT23 depends on its central IPT domain. ^{myc}SPT23^{HA} and ^{myc}SPT23$^{\Delta IPT}$ lacking the IPT domain were expressed in yeast cells, and the proteins p120 and p90 were detected by Western analysis using anti-myc antibodies.

(C) SPT23 p90 is peripherally attached to internal membranes. Whole cell extracts ("T") of wild-type cells expressing ^{myc}SPT23 were separated into soluble ("S") and membrane ("P") fractions by centrifugation. Membrane fractions were further incubated with lysis buffer, carbonate buffer of pH 11.3, or 0.1% SDS. SPT23 was detected by Western analysis using anti-myc antibodies. As a control, fractionation of the ER membrane protein DPM1 was monitored in parallel.

(D) SPT23 p90 is tightly associated with membranes. Membrane fractions of ^{myc}SPT23-expressing cells were prepared as above and incubated in buffers containing NaCl in concentrations between 0.15 M and 2.5 M. Soluble proteins were separated by centrifugation. Proteins of the membrane fraction were analyzed by anti-myc Western blots as described above.

(E) Coimmunoprecipitation of processed SPT23 p90 with SPT23 p120. Membranes of wild-type cells expressing ^{myc}SPT23^{HA} were purified as described above. SPT23 p120 was specifically immunoprecipitated after solubilization with N-dodecyl-maltoside by monoclonal anti-HA antibodies. Both SPT23 p120 and coimmunprecipitated SPT23 p90 were subsequently detected in anti-myc Western analysis. Total extract ("T") was 10% of the volume used for the immunoprecipitation; control lane "C" shows the immunoprecipitation with IgG.

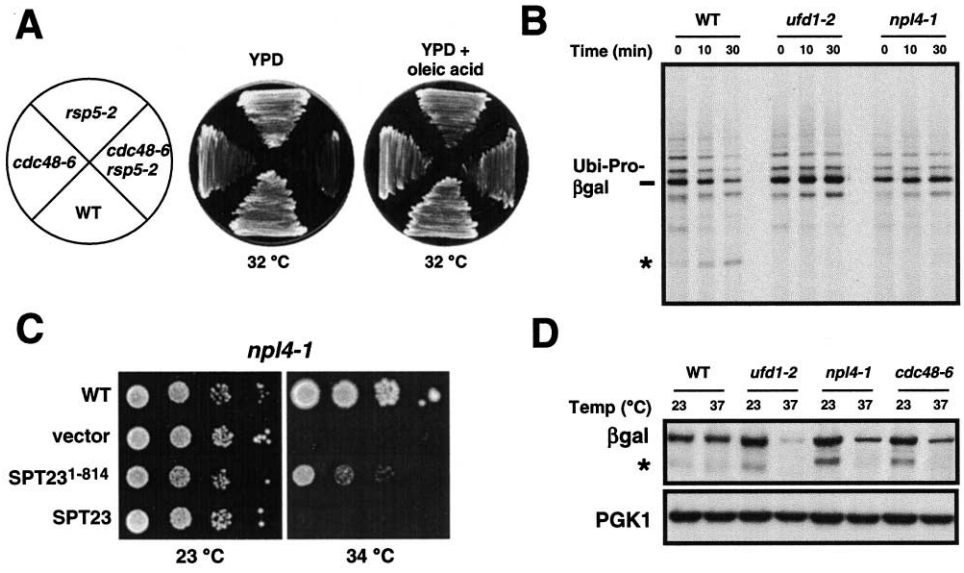

Figure 2. CDC48 and NPL4 Are Components of the OLE Pathway

(A) Growth of wild-type, *cdc48-6*, *rsp5-2*, and *cdc48-6 rsp5-2* double mutant cells on YPD plates and on YPD plates supplemented with 0.2% oleic acid (right) at 32°C. All strains are viable at 30°C.

(B) Pulse-chase analysis of ubiquitin-Proβ-galactosidase (Ubi-Proβgal) degradation in WT, *ufd1-2*, and *npl4-1* cells (grown at 37°C for 2 hr). Time points correspond to 0, 10, and 30 min. The asterisk marks the characteristic 90 kDa degradation product of Ubi-Proβgal.

(C) Colonies of serial diluted *npl4-1* cells transformed with either vector ("vector") or with plasmids resulting in overexpression of C-terminally truncated SPT23 ("SPT23$^{1-814}$"), or of full-length SPT23 ("SPT23"), respectively. In the top row, wild-type cells transformed with empty vector ("WT") are shown. The plates were incubated either at 23°C or at 34°C as indicated.

(D) Anti-β-galactosidase Western blot monitoring the expression of β-galactosidase under the control of the *OLE1* promoter in WT, *ufd1-2*, *npl4-1*, and *cdc48-6* cells that have been incubated either at 23°C or at 37°C for 3 hr. As a loading control, expression of PGK1 was monitored in parallel. The asterisk marks a breakdown product.

protein CDC48, an AAA-type ATPase that is thought to function in a chaperone-like manner (Golbik et al., 1999; Meyer et al., 2000). Using two-hybrid assays, we found that UFD1 indeed interacts with CDC48 (data not shown). Besides its function in the UFD pathway, CDC48 is known to be vitally important for membrane fusion (Latterich et al., 1995). Hence, not surprisingly, *cdc48-6* ts mutants could neither be rescued by overexpression of truncated SPT23 (which is able to rescue *ufd1-2* mutants; Hoppe et al., 2000), nor by adding oleic acid to the growth medium (data not shown). However, *cdc48-6* mutants are synthetically lethal with mutants (*rsp5-2*) in the gene for the ubiquitin ligase that mediates SPT23 processing, and viability is restored by adding oleic acid to the growth medium (Figure 2A), substantiating the idea that CDC48 plays a role in the OLE pathway.

Another candidate for the OLE pathway was NPL4, because recent reports have indicated that this protein interacts with UFD1 in two-hybrid assays as well (Uetz et al., 2000; Ito et al., 2001). NPL4, also encoded by an essential gene, was previously thought to function in nucleo/cytoplasmic transport (De Horatius and Silver, 1996; Nelson et al., 1993). By performing pulse-chase experiments, we found that *npl4-1* ts mutants stabilize the otherwise short-lived UFD substrate ubiquitin-β-galactosidase fusion protein (Ubi-Proβgal) similarly to *ufd1-2* and *cdc48-6* mutants (Figure 2B; Johnson et al., 1995; Ghislain et al., 1996). This finding identifies NPL4 as a bona fide component of the UFD pathway. We also observed that the thermosensitivity of *npl4-1* mutants could be rescued by overexpression of either C-terminally truncated SPT23 molecules, *OLE1*, or by the addition of oleic acid to the growth medium (Figure 2C and data not shown). Similar to *ufd1-2* mutants, *npl4-1* mutants could not be rescued by overexpression of full-length (i.e., membrane-bound) SPT23 (Figure 2C; Hoppe et al., 2000). From this finding, we assumed that UFD1 and NPL4 are required during or after SPT23 processing.

To unambiguously demonstrate that CDC48, UFD1, and NPL4 are indeed required for *OLE1* expression, we investigated the activity of the *OLE1* promoter by using an *OLE1* promoter-driven β-galactosidase-reporter construct. Indeed, when we shifted *cdc48-6*, *ufd1-2*, or *npl4-1* cells to their restrictive temperature, all three mutants expressed significantly lower levels of the reporter protein (Figure 2D).

CDC48, UFD1, and NPL4 Form a Complex in Yeast

Recently, Meyer et al. (2000) have shown that the mammalian ortholog of CDC48, p97, can form two alternative, mutually exclusive protein complexes. In association with p47, p97 can mediate membrane fusion events, and in conjunction with the mammalian homologs of yeast UFD1 and NPL4, p97 is thought to mediate other, ubiquitin-linked functions. Recent two-hybrid data suggest that a similar CDC48/UFD1/NPL4 complex might exist in budding yeast as well (Ito et al., 2001). Indeed, we found genetic interactions (synthetic lethality; Figure

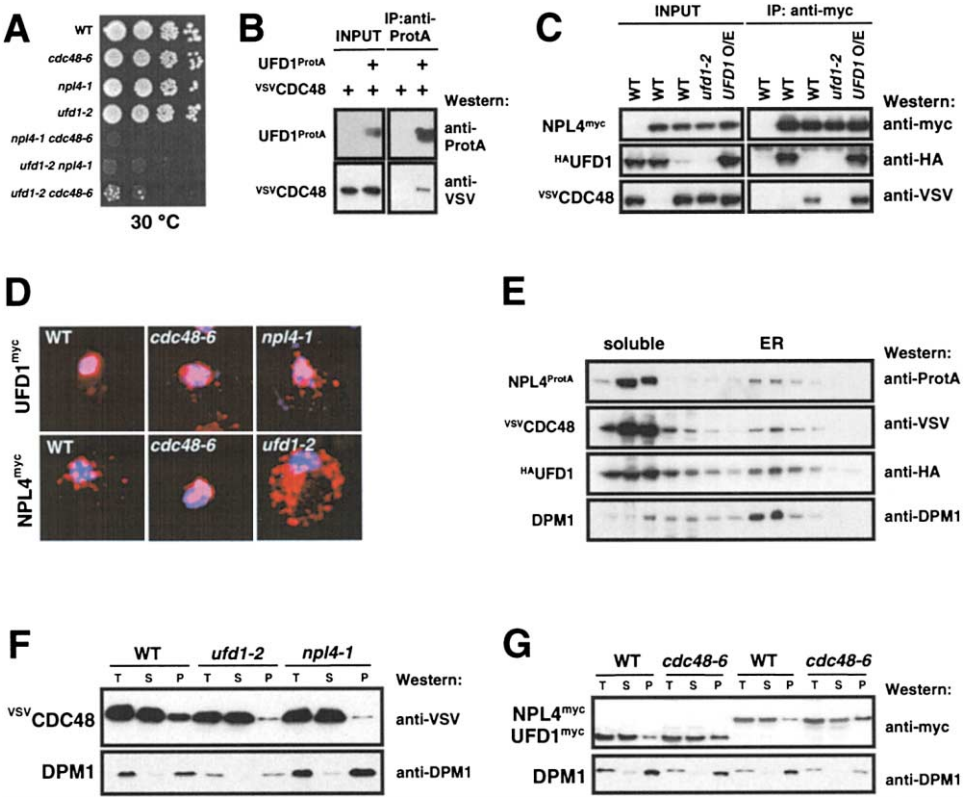

Figure 3. CDC48, UFD1, and NPL4 Form a Functional Unit in Yeast

(A) Serial dilutions of WT, *cdc48-6*, *npl4-1*, *ufd1-2*, and the respective double mutant cells at 30°C. All strains are viable at 23°C, the permissive temperature. Synthetic lethality is observed in the case of double mutants.

(B) Coimmunoprecipitation of VSV-tagged CDC48 expressed under its own promoter with chromosomally Protein A-tagged UFD1 using IgG-Sepharose beads. The left panel (INPUT; shown is 10% of the volume used for the immunoprecipitation) shows the level of the respective proteins in cells by Western blotting. The right panel shows the immunoprecipitates.

(C) Coimmunoprecipitation of HA-tagged UFD1 (^{HA}UFD1) and VSV-tagged CDC48 (^{VSV}CDC48) with myc-tagged NPL4 (NPL4^{myc}) using anti-myc antibodies. Wild-type (WT), *ufd1-2* mutants, and UFD1-overexpressing cells (*UFD1* O/E) were used, which express tagged proteins as shown in the "INPUT" panel. Note that the precipitated level of CDC48 is proportional to UFD1 protein levels. Protein levels (left) and immunoprecipitations (right) are displayed as above. The epitope-tagged proteins are functional because the constructs can complement the corresponding mutants.

(D) Subcellular localization of chromosomally myc-tagged UFD1 (UFD1^{myc}) and NPL4 (NPL4^{myc}) in wild-type, *cdc48-6*, and *ufd1-2* (in the case of NPL4^{myc}) or *npl4-1* (in the case of UFD1^{myc}) cells as determined by anti-myc immunofluorescence microscopy and deconvolution imaging. UFD1 and NPL4 are shown in red; DNA was visualized by the blue DAPI stain.

(E) Fractions of sucrose gradients from cells coexpressing NPL4^{ProtA}, ^{VSV}CDC48, and ^{HA}UFD1. Proteins were detected by Western blotting. ER-containing fractions were monitored by antibodies specific for the ER-marker protein DPM1.

(F) Subcellular fractionation of ^{VSV}CDC48 and (G) UFD1^{myc} and NPL4^{myc} in wild-type or in the indicated mutant cells. As a control, immunoreactivity against the ER membrane protein DPM1 was used. Mutant cells were grown at 37°C for 2 hr.

3A) between the corresponding genes. We also investigated physical interaction between these proteins by coimmunoprecipitation. As seen in Figure 3B, precipitation of tagged UFD1 coprecipitated tagged CDC48. Furthermore (Figure 3C), precipitation of tagged NPL4 coprecipitated UFD1 and CDC48. Importantly, CDC48 did not coprecipitate with NPL4 when we used an *ufd1-2* mutant at its restrictive temperature. But, conversely, when we overexpressed UFD1 in cells, the amount of tagged CDC48 that coprecipitates with NPL4 significantly increased. This indicates that the interaction of NPL4 with CDC48 might be indirect and that it is mediated by UFD1. Together, our data demonstrate that interactions of CDC48/p97 are conserved from yeast to mammals and that an oligomeric complex, designated CDC48$^{UFD1/NPL4}$, appears to be a functional unit.

The CDC48$^{UFD1/NPL4}$ Complex Associates with Membranes

CDC48 localizes to the cytosol and the nucleus, and it may shuttle between these compartments (Madeo et al., 1998). By immunofluorescence microscopy (Figure 3D) we observed that UFD1 (UFD1^{myc}) accumulates predominantly inside the nucleus of wild-type cells. In contrast, NPL4 (NPL4^{myc}) exhibits a more patchy localization adjacent to, or inside of, the nucleus, consistent with previous findings (De Horatius and Silver, 1996) that suggested an accumulation of NPL4 at the nuclear envelope or the ER. The localization of UFD1^{myc} was not significantly altered in either *cdc48-6* or *npl4-1* strains, but we observed a striking, broadly cytoplasmic distribution of NPL4^{myc} in *ufd1-2*. In contrast, the *cdc48-6* mutation had only a moderate influence on the intracel-

lular localization of NPL4, but the protein appeared more evenly distributed at the nucleus compared to wild-type cells. These data suggest that UFD1, but not CDC48, is the major determinant for the normal intracellular localization of NPL4.

A notable fraction of the cytosolic pool of CDC48 was reported to associate with ER membranes (Latterich et al., 1995). When we separated whole cell extracts on sucrose gradients (Figure 3E), we observed that CDC48, UFD1, and NPL4 cofractionated and that a significant fraction of all three proteins copurified with the ER-marker protein DPM1. Furthermore, we detected notable levels of CDC48, UFD1, and NPL4 in purified microsomal pellet fractions (Figures 3F and 3G). Importantly, association of CDC48 with the membrane was reduced in both *ufd1-2* and *npl4-1* ts mutant strains (Figure 3F). In contrast, membrane associations of UFD1 and NPL4 were consistently found to be slightly increased in *cdc48-6* mutant cells (Figure 3G). Together, these data indicate that the $CDC48^{UFD1/NPL4}$ complex associates with membranes and, importantly, that UFD1 and NPL4 significantly stimulate or mediate the association of CDC48 with the membrane. These data also suggest that CDC48 may release UFD1 and NPL4 proteins from the membrane. UFD1 and NPL4 could be washed off the membrane with either 0.5 M NaCl or a high pH buffer (data not shown), indicating that they are peripherally attached to membranes, however less tightly than SPT23 p90.

Inactivation of $CDC48^{UFD1/NPL4}$ Leads to Inactive, Mislocalized p90

We speculated that the association of $CDC48^{UFD1/NPL4}$ with membranes might be mediated through interaction with membrane-bound substrates and that membrane-bound SPT23 might be a candidate. Because overexpression of C-terminally truncated, but not membrane-bound, SPT23 molecules could suppress *ufd1-2* and *npl4-1* mutant phenotypes (see above), $CDC48^{UFD1/NPL4}$ might function during or downstream of the processing reaction. SPT23 processing requires the activities of the RSP5 ubiquitin ligase and the proteasome, and, accordingly, p90 levels are virtually absent in the corresponding mutants (Hoppe et al., 2000; Figure 4A). In striking contrast, apparently normal levels of p90 were generated in *ufd1-2*, *npl4-1*, and *cdc48-6* mutants after shift to their restrictive temperatures (Figures 4A and 4B). However, because these mutants are defective in *OLE1* transcription, the p90 molecules produced in these cells must be inactive. This result suggests that $CDC48^{UFD1/NPL4}$ is required for a p90-activation step downstream of the processing reaction. It is possible, however, that $CDC48^{UFD1/NPL4}$ may play an additional, nonessential role in SPT23 processing, as we also observed slightly higher p120 levels in these cells (Figures 4A and 4B). Intriguingly, a significant fraction of p120 accumulated as mono- or, more rarely, diubiquitinated species in these mutants (Figures 4A–4C), indicating that mono- or oligoubiquitination, rather than multiubiquitination, precedes SPT23 processing (see Discussion).

One possible function of $CDC48^{UFD1/NPL4}$ could be nuclear targeting of p90. To investigate the intracellular localization of p90 in these mutants, we followed the N-terminal myc-epitope of the $^{myc}SPT23^{HA}$ molecule by immunofluorescence microscopy. As has been observed previously (Hoppe et al., 2000), myc-labeled SPT23 primarily accumulated in the nucleus of wild-type cells. In striking contrast, $^{myc}SPT23$ failed to enter the nucleus of *cdc48-6*, *ufd1-2*, and *npl4-1* mutant cells but appeared to localize in the nuclear periphery or the ER at the nonpermissive temperature (Figure 4D). This defect was not due to a general block in nuclear transport, because other nuclear proteins were correctly targeted in these cells (Figure 4E). Most importantly, soluble $SPT23^{\Delta TM}$ also localized to the nucleus in these cells (Figure 4E).

CDC48 Mobilizes SPT23 p90 In Vitro

We hypothesized that $CDC48^{UFD1/NPL4}$ is needed to mobilize p90 for nuclear targeting, and developed an in vitro mobilization assay. We used constant amounts of microsomal membranes (which contained UFD1 and NPL4; see above) prepared from yeast cells that expressed $^{myc}SPT23$. We added protein extracts of yeast CDC48-expressing insect cells to these fractions, incubated the mixtures for 30 min at 27°C in the presence of ATP and proteasome inhibitors, and separated soluble and membrane-associated proteins by centrifugation. The amount of immunoreactive protein released from the pellet fraction into the supernatant was monitored by Western blotting. As shown in Figure 5A, CDC48 appears to stimulate the release of p90 from membranes. We also used purified HIS-tagged CDC48 ($^{HIS}CDC48$) and found that this protein was fully active in this assay as well (Figure 5B). Importantly, p90 mobilization by purified CDC48 did not occur when we used membrane fractions from *npl4-1* mutants grown at their nonpermissive temperature (Figure 5B), indicating that the observed mobilization activity is physiologically relevant and depends on the $CDC48^{UFD1/NPL4}$ machinery. When we added either apyrase to deplete the wild-type extract from ATP, or N-ethylmaleimid (NEM), the ability of CDC48 to mobilize p90 was completely blocked (Figure 5A). This result confirms that CDC48 is the mobilizing activity, because CDC48 is known to be an NEM-sensitive ATPase (Fröhlich et al., 1995), similar to other members of this protein family, including NSF, the NEM-sensitive factor involved in membrane fusion.

CDC48, a Ubiquitin-Selective Chaperone

The aforementioned data suggest that $CDC48^{UFD1/NPL4}$ unravels p90 from its interacting p120 partner protein. But does the enzyme complex directly bind the substrate? We were unable to detect binding of SPT23 to CDC48, UFD1, or NPL4 by two-hybrid assays (data not shown), but we asked whether we could detect interaction by coimmunoprecipitation. For these assays, we used cells that coexpressed tagged SPT23 ($^{myc}SPT23$) and tagged CDC48 ($^{VSV}CDC48$) and specifically immunoprecipitated p90 from the soluble fraction. For control experiments we used $^{VSV}CDC48$-expressing cells that coexpressed $^{myc}SPT23^{\Delta TM}$, the soluble variant lacking the transmembrane domain. As shown in Figure 6A, we could coimmunoprecipitate $^{VSV}CDC48$ with myc-tagged p90, indicating that the two proteins indeed interact. Strikingly, however, immunoprecipitates of $^{myc}SPT23^{\Delta TM}$ did not contain

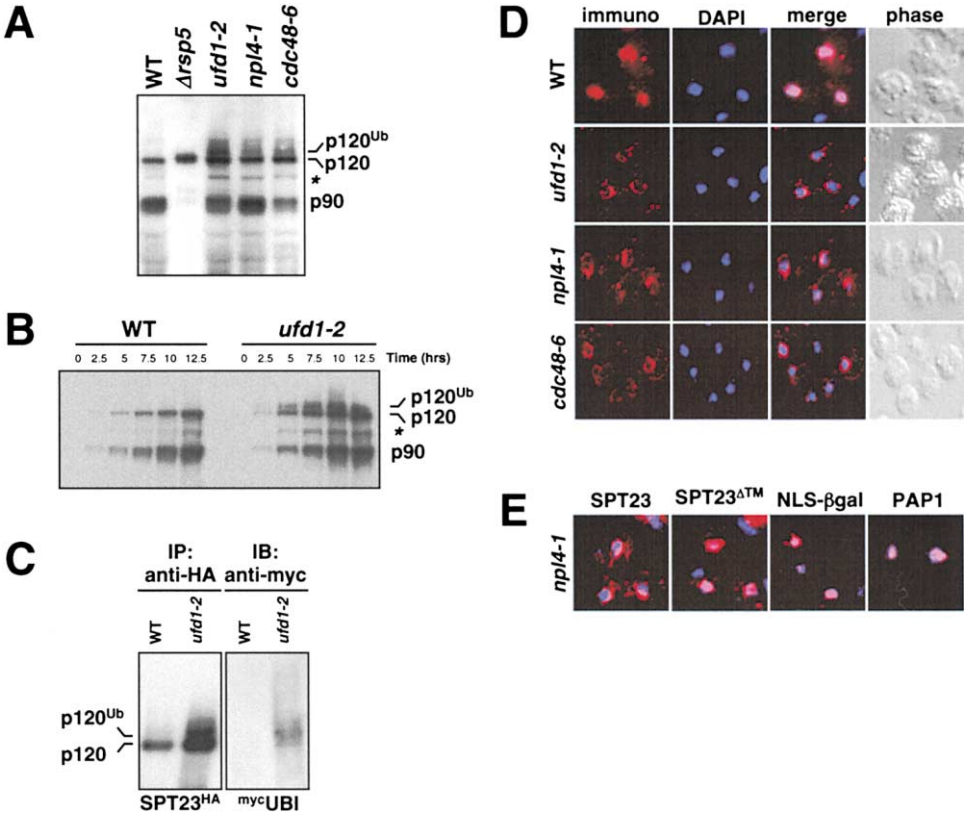

Figure 4. CDC48$^{UFD1/NPL4}$ Is Required for Nuclear Targeting of SPT23

(A) Steady-state levels of SPT23 p120 and p90 in WT and mutant cells. WT, *rsp5*Δ, *ufd1-2*, *npl4-1*, and *cdc48-6* cells were incubated at 37°C for 2 hr (similar results were obtained when cells were grown for 5 hr at 37°C). In the *ufd1-2*, *npl4-1*, and *cdc48-6* mutant strains, SPT23 p120 runs as a short ladder of bands with the upper bands representing mono and oligo-ubiquitinated species. The asterisk marks a crossreactivity of the anti-myc antibody.

(B) The CDC48$^{UFD1/NPL4}$ complex is not essential for SPT23 processing. WT and *ufd1-2* cells were pregrown on raffinose-containing medium supplemented with 0.2% oleic acid at 23°C. Cells were shifted to the nonpermissive temperature of *ufd1-2* cells (37°C), and ^{myc}SPT23^{HA} (under the control of the *GAL1* promoter) was induced by galactose. The medium was supplemented with oleic acid to restore viability of *ufd1-2* mutant cells. Cells were harvested after the indicated times and analyzed for SPT23 by Western blotting.

(C) Accumulation of ubiquitinated p120 in *ufd1-2* cells. HA-tagged SPT23 (SPT23^{HA}) was immunoprecipitated from wild-type (WT) and *ufd1-2* cells, which coexpress myc-tagged ubiquitin (^{myc}UBI) using HA-specific antibodies and probed by Western blotting with HA- (left panel) or myc-specific (right panel) antibodies.

(D) Intracellular localization of ^{myc}SPT23 in WT, *ufd1-2*, *npl4-1*, and *cdc48-6* cells as determined by anti-myc immunoflurescence microscopy and deconvolution imaging. SPT23 is shown in red, DNA in blue.

(E) Intracellular localization of ^{myc}SPT23, ^{myc}SPT23$^{\Delta TM}$, NLS-βgal, and PAP1^{myc} (poly-A polymerase) in *npl4-1* cells. The respective proteins detected by anti-myc or anti-βgal antibodies are shown in red, DNA in blue.

significant amounts of ^{VSV}CDC48, indicating that CDC48 has no affinity for this protein.

A significant difference between p90 and SPT23$^{\Delta TM}$ is that the former was generated in vivo by RUP. Thus, we considered the possibility that p90 might have retained ubiquitin moieties after RUP. In contrast, SPT23$^{\Delta TM}$ does not carry this modification. As we have shown previously (Hoppe et al., 2000), p90 of total cell extracts is ubiquitinated, but this modification could have taken place after the processing reaction in order to turn over the transcription factor. To test whether SPT23 p90 is ubiquitinated at the membrane, we precipitated SPT23 from purified microsomal fractions and probed the proteins in Western blots with ubiquitin-specific antibodies. Indeed, we found a single immunoreactive species with the mobility of p90 (Figure 6B). This finding strongly suggests that SPT23 has retained the ubiquitin modification after processing (see above) and that the 90 kDa species is in fact a conjugate of ubiquitin with an SPT23-derived polypeptide. In contrast to our previous studies where we used myc-tagged ubiquitin for detection (Hoppe et al., 2000), SPT23 p120 was not found to be significantly modified by ubiquitin at steady state. The observed difference can be explained by the fact that the myc-tag on ubiquitin is known to slow down ubiquitin-dependent reactions (Ellison and Hochstrasser, 1991). This suggests that RUP might be partially impaired by the ubiquitin variant, thereby leading to the previously observed accumulation of modified p120. As mentioned above, p120 accumulated as a mono- or oligoubiquitinated species in *cdc48*, *ufd1*, and *npl4* mutants (Figure 4A).

The different behavior of SPT23 p90 and SPT23$^{\Delta TM}$ with respect to CDC48 interaction could be due to the

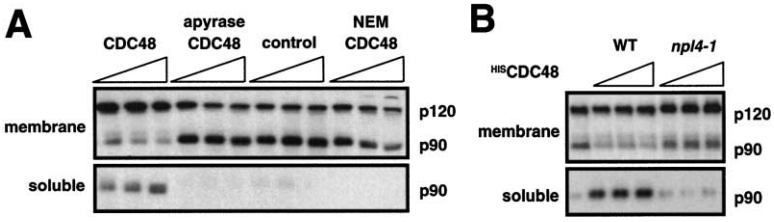

Figure 5. Mobilization of SPT23 p90 by CDC48 In Vitro

(A) Requirement for CDC48. Membrane fractions of wild-type cells expressing ^{myc}SPT23^{HA} were incubated at 30°C in the presence of ATP, PMSF, and proteasomal inhibitor MG132 with increasing amount (0.1, 0.2, 0.6 μg/μl protein) of extracts of baculovirus-infected cells expressing yeast CDC48. CDC48 was incubated either with buffer, apyrase, or a combination of NEM and EDTA. As control, an extract of baculovirus-infected cells, which does not express yeast CDC48, was added. Soluble proteins were separated from membrane-bound proteins by high-speed centrifugation. Fractions of both samples were analyzed for SPT23 by anti-myc immunoblots directed against an N-terminal myc-epitope. Note that SPT23 p90 appears in the soluble phase at the expense of p90 of the membrane fraction. No SPT23 p120 could be detected in the soluble fractions, indicating that microsomes have remained intact.

(B) Requirement for NPL4. The mobilization assay using increasing concentrations (10, 20, 60 ng/μl protein) of bacterially expressed, purified ^{HIS}CDC48 was done as above with membrane fractions from wild-type cells and from *npl4-1* mutant cells.

ubiquitin modification of p90. To test whether CDC48 has the general property of interacting selectively with ubiquitinated proteins, we again took advantage of the UFD pathway. The UFD substrate Ubi-Proβgal (see above) carries a single ubiquitin moiety fused to the *E. coli* protein β-galactosidase and requires the CDC48$^{UFD1/NPL4}$ machinery for degradation (Johnson et al., 1995; Ghislain et al., 1996, and see above). Ub-Met-βgal is a similar protein, but due to a single amino acid exchange (methionine instead of proline), the ubiquitin moiety of the fusion protein is cotranslationally removed by ubiquitin-C-terminal hydrolases. We immunoprecipitated both proteins with monoclonal anti-β-galactosidase antibodies from ^{VSV}CDC48-expressing cells and asked whether ^{VSV}CDC48 coprecipitates with one of these two proteins. Intriguingly, ^{VSV}CDC48 was found in the precipitate of the UFD substrate, but not in that of the nonubiquitinated control protein, suggesting that CDC48's ability to bind substrates is indeed linked to the ubiquitin modification.

Because the above experiment was done with total cell extracts, binding of CDC48 to the ubiquitinated substrate could have been mediated indirectly through other proteins. To exclude this possibility, we purified ^{HIS}CDC48, glutathione S-transferase (GST), and a ubiquitin-GST fusion protein (Ubi-GST; Koegl et al., 1999), and asked whether these purified proteins interact in vitro. As shown in Figure 6D, glutathione beads pulled down GST and Ubi-GST with roughly similar efficiencies. Intriguingly, however, ^{HIS}CDC48 was only efficiently pulled down with the Ubi-GST fusion protein and not with GST. Binding of Ubi-GST to CDC48 was indeed due to the ubiquitin moiety present in the fusion protein, because the efficiency of binding was significantly reduced if high concentrations of free ubiquitin were present in the pull-down assay (Figure 6D). Together, these findings

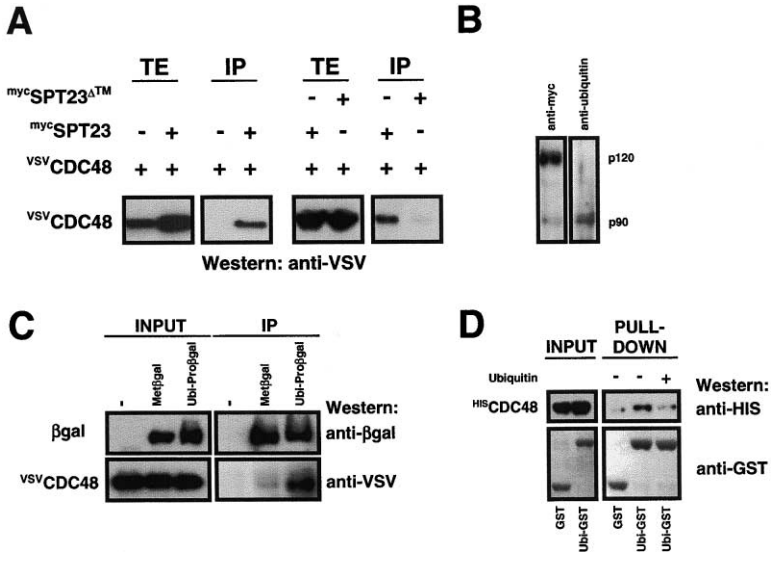

Figure 6. CDC48 Preferentially Interacts with Ubiquitinated Proteins

(A) Coimmunoprecipitation of ^{VSV}CDC48 with ^{myc}SPT23 but not with ^{myc}SPT23^{ΔTM}. Immunoprecipitations were performed with antibodies directed against the N-terminal myc-epitope of the respective SPT23 proteins. Both total cell extracts ("TE"; 10% of the volume used for the immunoprecipitation) and immunoprecipitation reactions ("IP") were analyzed for CDC48 by anti-VSV immunoblots.

(B) Ubiquitination of membrane-associated SPT23 p90. Membranes of wild-type cells expressing ^{myc}SPT23^{HA} were prepared as described above. Solubilized SPT23 was immunoprecipitated and subsequently analyzed for ubiquitination by anti-ubiquitin antibodies.

(C) Coimmunoprecipitation of ^{VSV}CDC48 by Ubi-Proβgal but not by Metβgal in ^{VSV}CDC48-overexpressing cells. Ubi-Proβgal and Metβgal were immunoprecipitated from cell lysates by monoclonal anti-βgal antibodies. Coimmunoprecipitated ^{VSV}CDC48 was detected by anti-VSV antibodies. The left panel shows the in vivo protein levels by Western blotting (INPUT; 10% of the volume used for the immunoprecipitation). The right panel shows the immunoprecipitates (IP) by using βgal-specific antibodies. Immunoprecipitated proteins were detected by Western blotting using βgal- and VSV-specific antibodies.

(D) Purified CDC48 binds Ubi-GST but not GST in vitro. Bacterially expressed HIS-tagged CDC48 (^{HIS}CDC48), GST, and a ubiquitin-GST fusion protein (Ubi-GST) were purified to homogeneity and assayed for binding by a pull-down using glutathione beads (the volume used for the input is identical to that used for the pulldown). Bound material was detected by Western blotting using anti-HIS and anti-GST antibodies. Binding of ^{HIS}CDC48 to Ubi-GST was reduced when an access of free ubiquitin was present in the assay.

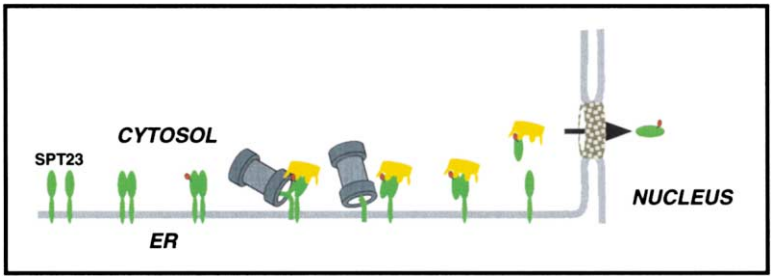

Figure 7. Hypothetical Model of SPT23 Processing and Mobilization

SPT23 (green) at the ER might dimerize via the IPT domains before RSP5-mediated monoubiquitination (red ball). Processing of SPT23 by the proteasome (gray barrel) leads to p90, which is associated with an uncleaved p120 partner molecule. Processing might be assisted by CDC48$^{UFD1/NPL4}$ (yellow crown). The processed p90 has retained its ubiquitin modification, and CDC48$^{UFD1/NPL4}$ removes p90 from its p120 partner. Subsequently, p90 can enter the nucleus to drive *OLE1* transcription.

strongly suggest that CDC48 is a chaperone-like enzyme with a pronounced specificity toward ubiquitinated proteins.

Discussion

The Conserved CDC48 Chaperone-like Machinery

CDC48 belongs to the large family of AAA proteins that are believed to function similar to chaperones. Members of this family include NSF, involved in vesicle fusion, and the RPT subunits of the 19S cap of the proteasome, which assist in protein degradation. CDC48 is particularly interesting, as it seems to mediate a variety of cellular activities: it is required for cell division, homotypic fusion of ER membranes, and degradation of certain artificial ubiquitin-fusion proteins (the UFD pathway). How this remarkable functional diversity of the enzyme may be achieved has been revealed recently (Meyer et al., 2000). The mammalian protein p97 (also known as VCP), the putative ortholog of CDC48, was shown to assemble into two distinct complexes. In alliance with p47, the p97 protein mediates membrane fusion (and possibly cell division; Kondo et al., 1997). Yet, if assembled with UFD1 and NPL4, the complex is inactive in membrane fusion and appears to mediate other functions. Homologs of these cofactors exist in yeast, and we now show that yeast CDC48 can assemble with UFD1 and NPL4 to form a complex designated CDC48$^{UFD1/NPL4}$. CDC48 additionally interacts with the yeast protein SHP1, the presumed ortholog of p47 (S. Braun et al., unpublished data). These findings indicate that yeast cells also possess two alternative, functionally distinct complexes: CDC48$^{UFD1/NPL4}$ and CDC48^{SHP1}.

Function of CDC48$^{UFD1/NPL4}$ in the OLE Pathway

Whereas the role of CDC48/p97 in membrane fusion events is well studied (Patel et al., 1998), the function of the alternative CDC48$^{UFD1/NPL4}$ complex has remained enigmatic. Our work now shows that CDC48$^{UFD1/NPL4}$ is essential for the OLE pathway and that this function is important for cell viability. Specifically, we found that the CDC48$^{UFD1/NPL4}$ machinery catalyzes, by an ATP-driven mechanism, the dissociation of membrane-bound SPT23 p90-p120 dimers, and, subsequently, the mobilization of p90 from the membrane (Figure 7). Notably, p90 remains bound to the CDC48 chaperone after it is freed from its partner, thereby preventing the reassociation of p90 with another p120 molecule at the membrane. Although CDC48$^{UFD1/NPL4}$ does not seem to be required for SPT23 processing (Figure 4B), our data indicate that the enzyme might facilitate this reaction. This suggests that CDC48$^{UFD1/NPL4}$ may associate with the SPT23 dimer at the membrane already prior to or during processing and that the enzyme leaves the membrane in a complex with p90 (Figure 7).

While this work was under review, Silver and coworkers (Hitchcock et al., 2001) published related work on the essential function of the CDC48$^{UFD1/NPL4}$ complex in *OLE1* activation. However, these authors report that processing of N-terminal GFP fusions of SPT23/MGA2 is blocked in *cdc48*, *ufd1*, and *npl4* mutants at 25°C. But these mutants are viable and proficient in *OLE1* expression at this temperature, demonstrating that processing of endogenous, wild-type SPT23/MGA2 is not blocked under these conditions. We suggest that the difference in the two substrates could be due to the bulky GFP moiety, which may interfere with precursor dimerization, an essential prerequisite of precursor processing (Figure 1B).

Our finding that SPT23 processing involves a tight association of two SPT23 molecules may offer an explanation for the puzzling phenomenon of limited proteolysis by proteasomes. Ubiquitin/proteasome-dependent processing of both SPT23 p120 and NF-κB p105 is thought to be initiated by an internal cleavage (Hoppe et al., 2000; Lin and Ghosh, 1996). This strongly argues for a model in which the substrate reaches the active sites within the proteasome by hairpin formation (Hoppe et al., 2000). Both SPT23 and p105 form homodimers via their IPT domains, which are located N-terminally of the initial cleavage site (Figure 1 and Lin et al., 2000). These interactions may be strong enough to prevent the translocation of the N-terminal domains into the cavity of the proteasome. As a result, only sequences C-terminal of the initial cut are degraded by the proteasome, whereas the N-terminal parts remain intact. Indeed, previous findings have indicated that the proteasome is unable to degrade tightly folded protein domains (Johnston et al., 1995; Lee et al., 2001), indicating that limited proteolysis by the proteasome might be more frequent than previously expected.

Similarity to NSF

Substrate binding of CDC48 is mediated by its N-terminal, so-called N-domain. Interestingly, this domain harbors a region that is structurally similar to UFD1 (Golbik et al., 1999), suggesting that the enzyme may gain additional substrate binding sites through interaction with UFD1. Active CDC48/p97 is organized as a homohexameric ring. Recent structural studies have shown that

this ring undergoes dramatic ATP-dependent conformational changes (Zhang et al., 2000; Rouiller et al., 2000), indicating that the unfolding or unraveling activity of CDC48 may be brought about by these movements. The protein stoichiometry within $CDC48^{UFD1/NPL4}$ is presently not known, but it is conceivable that perhaps six or three pairs of UFD1/NPL4 may assemble on top of the hexameric CDC48 ring. This arrangement may lead to an amplification of the movements of the complex and may empower the unfoldase activity of the enzyme.

Interestingly, the activity of $CDC48^{UFD1/NPL4}$ toward SPT23 p90/p120 dimers strikingly resembles the activity of NSF in vesicle fusion events. The substrates of NSF are the membrane-bound SNARE proteins, which, after vesicle fusion, are tightly tethered together through their N-terminal coiled-coil domains. Notably, NSF requires the adaptor protein α-SNAP for SNARE binding (Clary et al., 1990), which can be considered as the functional equivalent to UFD1/NPL4. Like CDC48, NSF forms homohexameric rings, which undergo ATP-driven conformational changes (Yu et al., 1999). This movement is used to unravel the SNAREs at the membrane, an activity needed to recycle the SNARE proteins for further fusion reactions (Söllner et al., 1993). This interesting parallel between CDC48 and NSF strongly suggests that oligomeric AAA proteins in general may have unraveling functions and catalyze protein disassembly.

CDC48, a Ubiquitin-Selective Chaperone

Our data demonstrate that CDC48 has a pronounced specificity toward ubiquitin. We have shown that CDC48 binds p90, a ubiquitinated polypeptide, but that it does not interact with the unmodified soluble protein $SPT23^{\Delta TM}$. By using β-galactosidase-specific antibodies, we also showed that CDC48 coprecipitates with ubiquitinated β-galactosidase, but not with the unmodified form of the same protein. Importantly, by using purified proteins, we could show that binding of CDC48 to ubiquitinated substrates (GST) is direct and not mediated through other proteins. These findings are supported by a previous report that suggested that the mammalian ortholog of CDC48, p97, also selectively binds the ubiquitinated form of IκB (Dai et al., 1998). What might be the significance of a chaperone that can discriminate between ubiquitinated and nonubiquitinated proteins? The key function of CDC48/p97 is to disassemble protein complexes. If a ubiquitinated protein is part of a protein assembly, CDC48 will selectively remove the modified subunit from the complex. In the OLE pathway, this mechanism is used to mobilize p90 for its nuclear function. In other cases (e.g., IκB), this specificity might be utilized to selectively degrade a specific subunit of a multimeric complex. Moreover, we have recently discovered that $CDC48^{UFD1/NPL4}$ is also involved in ER-associated degradation (ERAD) (S. Braun et al., unpublished data). In this scenario, the CDC48 machinery might selectively deliver ubiquitinated proteins for proteasomal degradation.

The specificity for ubiquitinated substrates might be restricted to the $CDC48^{UFD1/NPL4}$ complex, however. The formation of the alternative complex, $CDC48^{SHP1}$, is mediated by interaction of the C-terminal UBX domain of SHP1(p47) with the N-domain of CDC48 (A. Buchberger, R. Albang, S. Thoms, and S.J., unpublished data). Intriguingly, the UBX domain, despite of a lack of apparent sequence similarity, has a three-dimensional structure strikingly similar to that of ubiquitin (Buchberger et al., 2001). This observation suggests that the N-domain of CDC48 mediates association with ubiquitin conjugates and that SHP1 (p47), in order to form the alternative $CDC48^{SHP1}$ complex, can occupy this site through molecular mimicry. Because of these findings, we propose that the N-domain of CDC48 is able to bind a single ubiquitin fold. A hexameric CDC48 complex may thus bind up to six ubiquitin moieties of a multiubiquitinated substrate.

Several AAA proteins have N-domains analogous to CDC48. But whether other AAA proteins, in addition to CDC48, bind ubiquitin or ubiquitin-like folds is currently not clear. Candidates perhaps include other AAA proteins that are known to function within the ubiquitin/proteasome pathway, i.e., the six RPT subunits of the 19S cap of the proteasome. Similar to CDC48, these subunits are thought to form a hexameric ring, which, in this case, is assembled on top of the 20S proteolytic particle. It seems attractive to speculate that these proteins might have a preference for ubiquitin moieties by which they may contribute to the ubiquitin specificity of the proteasome.

Experimental Procedures

Cloning and Yeast Techniques

Standard yeast protocols were used (Guthrie and Fink, 1991). Media and plates were supplemented with 0.2% oleic acid dissolved in Nonidet P40 (0.2% final) where indicated. Strains were derivatives of DF5 (Hoppe et al., 2000). *ufd1-2* was constructed by a PCR-based strategy. *npl4-1* was a gift of P. Silver, *cdc48-6* of K.U. Fröhlich, and *rsp5-2* of B. Seraphin. Mutations were introduced into the DF5 background by repetitive mating and tetrade dissection. $^{myc}SPT23^{HA}$ and $^{myc}SPT23^{\Delta TM}$ were described previously (Hoppe et al., 2000). ^{VSV}CDC48 (in *YEplac112*) and ^{HA}UFD1 (in *YEplac181*) were expressed under the control of their own promoters. When indicated, genes were chromosomally tagged with myc (3 copies) and Protein A-epitopes by the method of Knop et al., 1999. GST and Ubi-GST were expressed in *E. coli* cells and purified to homogeneity as described (Koegl et al., 1999). The open reading frame of CDC48 was cloned into pQE32 to generate ^{HIS}CDC48. ^{HIS}CDC48 was purified to homogenity from *E. coli* BL21 (DE3)-RIL according to the manufacturer's instructions (Stratagene).

Membrane Fractionation

Fractionation of $^{myc}SPT23^{HA}$ was performed as described (Hoppe et al., 2000), with the exception that lysis buffer contained 1 mM EDTA and 2 mM PMSF. In brief, total cell extract was fractionated in crude membrane and cytosolic fraction by centrifugation at 20,000 $\times$ g. Soluble and pellet fractions were probed by Western blotting with polyclonal anti-myc antibodies (Santa Cruz Biotechnology) or with anti-DPM1 antibodies (Molecular Probes). To test the stability of membrane-bound SPT23 p90, the membrane fraction was washed with lysis buffer containing NaCl in concentrations between 0.15 and 2.5 M.

Immunoprecipitation of Membrane-Bound SPT23

WT and *ufd1-2*, *npl4-1*, and *cdc48-6* strains expressing $^{myc}SPT23^{HA}$ were grown in YPGal to OD_{600} of 0.6 and shifted to 37°C for 2 hr. Membranes were prepared as described above. Membrane-bound proteins were solubilized by incubation in 150 μl lysis buffer containing 0.4% N-dodecyl-maltoside for 90 min on ice. The reactions were diluted with detergent-free lysis buffer to a final concentration of 0.1% N-dodecyl-maltoside and incubated with 2 μg monoclonal anti-HA antibodies for 90 min at 4°C. Protein G-agarose beads

(Roche) were added for another 60 min at 4°C, subsequently washed five times with lysis buffer containing 0.1% N-dodecyl-maltoside, and incubated in HU buffer (Knop et al., 1999) for 15 min at 65°C. Proteins were separated on 4%–12% gradient gels and analyzed by anti-myc and anti-HA immunoblots, respectively.

Immunoprecipitation of CDC48 Interactors

Yeast strains expressing ^{myc}SPT23^{HA} or Ubiquitin-Proβgal, and ^{VSV}CDC48 were grown to an OD_{600} of 0.6. Thermosensitive strains were shifted to the restrictive temperature for 3 hr. Cells were lysed with glass beads and lysis buffer A (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 2 mM PMSF). Extracts were centrifuged at 20,000 × g and subjected to immunoprecipitation with myc-antibodies (Santa Cruz) directed against SPT23 or monoclonal β-galactosidase-antibodies (Promega) in lysis buffer A with 0.1% Triton X-100. After 90 min, protein G agarose beads were added for a further 60 min at 4°C. Bound proteins were analyzed by immunoblots against the VSV-tag of CDC48 and the respective epitope-tag used for the immunoprecipitation.

GST Pull-Down Experiments

Equal amounts of GST and Ubi-GST were coupled to glutathione beads and incubated with purified ^{HIS}CDC48 in binding buffer (25 mM Tris/HCl [pH 8], 150 mM NaCl, 2.5 mM MgCl2, 2.5 mM ATP) for 120 min at 4°C. Beads were washed carefully with binding buffer containing 0.05% Tween 20 and eluted by incubation in HU buffer. Bound proteins were detected after 4%–12% gradient gel electrophoresis and Western blotting using anti-HIS- (Dianova) or anti-GST-antibodies (Dianova).

Ubiquitination of Membrane-Bound SPT23

Membrane-bound proteins of WT or *ufd1-2* cells expressing SPT23^{HA} and myc-ubiquitin or WT cells expressing ^{myc}SPT23^{HA} were solubilized as described above. SPT23 was immunoprecipitated from this fraction as described above. Immunoprecipitated proteins were separated on 4%–12% gradient gels and analyzed by anti-myc or anti-ubiquitin immunoblots. Blots were stripped and subsequently analyzed for SPT23.

SPT23 Mobilization Assay

WT or *npl4-1* cells overexpressing ^{myc}SPT23^{HA} under galactose control were grown in YPGal to an OD_{600} of 1 and shifted for 2 hr to 37°C. Extracts were prepared by glass bead lysis in lysis buffer B (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, 1 mM MG132) followed by preparation of the membrane fraction by centrifugation at 20,000 × g, 4°C, for 30 min. Membranes corresponding to OD_{600} = 10 were washed with lysis buffer and then resuspended on ice in reaction buffer (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl2, 2 mM ATP, creatine phosphate, creatine kinase, 1 mM MG132, and 1 mM PMSF). CDC48 (either extract of baculovirus-infected insect cells overexpressing CDC48 or purified HIS-CDC48) that had been preincubated either in reaction buffer, apyrase-, or NEM/EDTA containing buffer was added in increasing concentrations. The reactions were initiated by transfer to 27°C for 30 min. To stop the reactions, samples were centrifuged at 20,000 × g at 4°C for 30 min; both the soluble and the pellet fraction were incubated in HU buffer at 65°C for 15 min, and analyzed by SDS PAGE and anti-myc Western blots.

Sucrose Gradient Centrifugation

Cells expressing NPL4^{ProtA}, ^{HA}UFD1, and ^{VSV}CDC48 were grown in synthetic medium to an OD_{600} of 1, incubated with zymolase 100T, and lysed with a Wheaton dounce tissue grinder in lysis buffer A (see above). Total lysate was loaded onto a 28%–54% sucrose gradient and centrifuged for 3.5 hr at 30,000 rpm. The gradient was fractionated, proteins were separated on 4%–12% gradient gels and analyzed with anti-ProtA, anti-HA, and anti-VSV-immunoblots. ER-containing fractions were identified by immunoreactivity against the ER protein DPM1.

Immunofluorescence Analyses

Localization of myc-epitope tagged UFD1, NPL4, and SPT23 was studied in WT and the respective mutant strains by indirect deconvolution immunofluorescence as previously described (Hoppe et al., 2000). WT strains expressing myc-tagged UFD1 or NPL4 were grown in YPD to an OD_{600} of 0.6. The WT strain containing *YIplac211-GAL::^{myc}SPT23^{HA}* was grown in YPGal to an OD_{600} of 0.6. Cells were fixed with formaldehyde for 60 min at room temperature. The myc-epitopes were detected by monoclonal antibodies (Santa Cruz) overnight at 4°C followed by incubation with Alexa546-labeled goat anti-mouse antibodies (Molecular Probes). DNA was stained with DAPI (Molecular Probes).

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References

Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell *100*, 391–398.

Buchberger, A., Howard, M.J., Proctor, M., and Bycroft, M. (2001). The UBX domain: a widespread ubiquitin-like molecule. J. Mol. Biol. *307*, 17–24.

Clary, D.O., Griff, I.C., and Rothman, J.E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell *61*, 709–721.

Dai, R.M., Chen, E., Longo, D.L., Gorbea, C.M., and Li, C.C. (1998). Involvment of valosin-containing protein, an ATPase co-purified with IκBα and 26S proteasome, in ubiquitin-proteasome-mediated degradation of IκBα. J. Biol. Chem. *273*, 3562–3573.

De Horatius, C., and Silver, P. (1996). Nuclear transport defects and nuclear envelope alterations are associated with mutation of the *Saccharomyces cerevisiae NPL4* gene. Mol. Biol. Cell *7*, 1835–1855.

Ellison, M.J., and Hochstrasser, M. (1991). Epitope-tagged ubiquitin. A new probe for analyzing ubiquitin function. J. Biol. Chem. *266*, 21150–21157.

Fröhlich, K.U., Fries, H.W., Peters, J.M., and Mecke, D. (1995). The ATPase activity of purified CDC48p from *Saccharomyces cerevisiae* shows complex dependence on ATP-, ADP-, and NADH-concentrations and is completely inhibited by NEM. Biochim. Biophys. Acta *1253*, 25–32.

Ghislain, M., Dohmen, R.J., Levy, F., and Varshavsky, A. (1996). Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. EMBO J. *15*, 4884–4899.

Golbik, R., Lupas, A.N., Koretke, K.K., Baumeister, W., and Peters, J. (1999). The Janus face of the archeal CDC48/p97 homologue VAT: protein folding *versus* unfolding. Biol. Chem. *380*, 1049–1062.

Guthrie, C., and Fink, G.R. (1991). Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology. (New York: Academic Press).

Hampton, R.Y., Gardner, R.G., and Rine, J. (1996). Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol. Biol. Cell *7*, 2029–2044.

Hitchcock, A.L., Krebber, H., Frietze, S., Lin, A., Latterich, M., and Silver, P.A. (2001). The conserved Npl4 protein complex mediates proteasome-dependent membrane-bound transcription factor activation. Mol. Biol. Cell *12*, 3226–3241.

Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H.D., and Jentsch, S. (2000). Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. Cell *102*, 577–586.

Hoppe, T., Rape, M., and Jentsch, S. (2001). Membrane-bound transcription factors: regulated release by RIP or RUP. Curr. Opin. Cell Biol. *13*, 344–348.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. USA *98*, 4569–4574.

Johnson, E.S., Ma, P.C., Ota, I.M., and Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. *270*, 17442–17456.

Johnston, J.A., Johnson, E.S., Waller, P.R., and Varshavsky, A. (1995). Methotrexate inhibits proteolysis of dihydrofolate reductase by the N-end rule pathway. J. Biol. Chem. *270*, 8172–8178.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast *15*, 963–972.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635–644.

Kondo, H., Rabouille, C., Newman, R., Levine, T.P., Pappin, D., Freemont, P., and Warren, G. (1997). p47 is a cofactor for p97-mediated membrane fusion. Nature *388*, 75–78.

Latterich, M., Fröhlich, K.U., and Schekman, R. (1995). Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. Cell *82*, 885–893.

Lee, C., Schwartz, M.P., Prakash, S., Iwakura, M., and Matouschek, A. (2001). ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. Mol. Cell *7*, 627–637.

Lin, L., and Ghosh, S. (1996). A glycine-rich region in NF-κB p105 functions as a processing signal for the generation of the p50 subunit. Mol. Cell. Biol. *16*, 2248–2254.

Lin, L., DeMartino, G.N., and Greene, W.C. (2000). Cotranslational dimerization of the Rel homology domain of NF-κB1 generates p50-p105 heterodimers and is required for effective p50 production. EMBO J. *19*, 4712–4722.

Madeo, F., Schlauer, J., Zischka, H., Mecke, D., and Fröhlich, K.U. (1998). Tyrosine phosphorylation regulates cell cycle-dependent nuclear localization of Cdc48p. Mol. Biol. Cell *9*, 131–141.

Meyer, H.H., Shorter, J.G., Seemann, J., Pappin, D., and Warren, G. (2000). A complex of mammalian UFD1 and NPL4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. EMBO J. *19*, 2181–2192.

Nelson, M.K., Kurihara, T., and Silver, P. (1993). Extragenic suppressors of mutations in the cytoplasmic C terminus of SEC63 define five genes in *Saccharomyces cerevisiae*. Genetics *134*, 159–173.

Patel, S.K., Indig, F.E., Olivieri, N., Levine, N.D., and Latterich, M. (1998). Organelle membrane fusion: a novel function for the syntaxin homolog Ufe1p in ER membrane fusion. Cell *92*, 611–620.

Rouiller, I., Butel, V.M., Latterich, M., Milligan, R.A., and Wilson-Kubalek, E.M. (2000). A major conformational change in p97 AAA ATPase upon ATP binding. Mol. Cell *6*, 1485–1490.

Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell *75*, 409–418.

Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., et al. (2000). A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature *403*, 623–627.

Yu, R.C., Jahn, R., and Brunger, A.T. (1999). NSF N-terminal domain crystal structure: models for NSF function. Mol. Cell *4*, 97–107.

Zhang, S., Skalsky, Y., and Garfinkel, D.J. (1999). MGA2 or SPT23 is required for transcription of the Δ9 fatty acid desaturase gene, *OLE1*, and nuclear membrane integrity in *Saccharomyces cerevisiae*. Genetics *151*, 473–483.

Zhang, X., Shaw, A., Bates, P.A., Newman, R.H., Gowen, B., Orlova, E., Gorman, M.A., Kondo, H., Dokurno, P., Lally, J., et al. (2000). Structure of the AAA ATPase p97. Mol. Cell *6*, 1473–1484.