

Cdc48/p97 Mediates UV-Dependent Turnover of RNA Pol II

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SUMMARY

Cdc48/p97 is an essential ATPase whose role in targeting substrates to the ubiquitin-proteasome system (UPS) remains unclear. Existing models posit that Cdc48 acts upstream of UPS receptors. To address this hypothesis, we examined the association of ubiquitin (Ub) conjugates with 26S proteasomes. Unexpectedly, proteasomes isolated from *cdc48* mutants contain high levels of Ub conjugates, and mass spectrometry identified numerous nonproteasomal proteins, including Rpb1, the largest subunit of RNA Pol II. UV-induced turnover of Rpb1 depends upon Cdc48-Ufd1-Npl4, Ubx4, and the uncharacterized adaptor Ubx5. Ubiquitinated Rpb1, proteasomes, and Cdc48 accumulate on chromatin in UV-treated wild-type cells, and the former two accumulate to higher levels in mutant cells, suggesting that degradation of Rpb1 is facilitated by Cdc48 at sites of stalled transcription. These data reveal an intimate coupling of function between proteasomes and Cdc48 that we suggest is necessary to sustain processive degradation of unstable subunits of some macromolecular protein complexes.

INTRODUCTION

Budding yeast Cdc48 is a member of the AAA (ATPases associated with various cellular activities) protein family. Cdc48 has been implicated in a plethora of functions that include cell-cycle regulation, membrane fusion, the stress response, and endoplasmic reticulum (ER)-associated degradation (ERAD) (Hirsch et al., 2009; Vembar and Brodsky, 2008). Its highly conserved mammalian counterpart, p97, has been additionally implicated in reformation of the nucleus (Ramadan et al., 2007), organelle biogenesis (Halawani and Latterich, 2006), myofibril organization (Janiesch et al., 2007), and the degradation of proteins such as Hif1- α (Alexandru et al., 2008) and HMG-CoA reductase (DeBose-Boyd, 2008).

Two underlying properties of Cdc48/p97 contribute to its myriad functions: its ATPase activity and the ability to bind ubiquitin (Ub) (Ye, 2006). Together, these activities are thought to

underpin a “segregase” function that separates ubiquitinated polypeptides from tightly bound partner proteins, enabling selective degradation of the former (Braun et al., 2002; Johnson et al., 1990). The Ub-binding activity of Cdc48 is synergistically enhanced by the binding of adaptors belonging to the UFD (Ub-fusion degradation) pathway (Johnson et al., 1995) and/or to the UBX (Ub regulatory X) family, members of which possess Ub-binding domains (Schuberth and Buchberger, 2008). The best-understood function of Cdc48 is in ERAD, where, in conjunction with its cofactors Ufd1 and Npl4, it extracts misfolded proteins from the ER membrane by virtue of its ATPase activity. The ubiquitinated substrates subsequently engage the Ub-associated (UBA) domain-containing receptors Rad23 and Dsk2, which in turn bind to the proteasome via their Ub-like (Ubl) domains, thereby delivering substrates for degradation (Raasi and Wolf, 2007).

The breadth of Cdc48's involvement in the ubiquitin-proteasome system (UPS) remains poorly understood. Whereas Cdc48 function clearly plays a prominent role in turnover of ER proteins (Jarosch et al., 2002; Rabinovich et al., 2002; Ravid et al., 2006; Ye et al., 2001), the connection between Cdc48 and the UPS was first discovered based on the requirement of Cdc48 and UFD proteins for turnover of non-ER, soluble reporter substrates (Ghislain et al., 1996). Fractionation studies and live imaging of Cdc48-GFP indicate that only a portion of yeast Cdc48 is peripherally bound to the ER/nuclear envelope, with most being partitioned between the cytosol and nucleus (Madeo et al., 1998)(Huh et al., 2003). Cdc48 has recently been shown to be required for the degradation of the cytosolic protein fructose-1,6-bisphosphatase (FBPase) (Barbin et al., 2010). Given the relative dearth of soluble (and physiological) UPS substrates, we wished to identify substrates whose degradation depends on Cdc48 and determine at what stage in their degradation Cdc48 is required.

Although there is general agreement that Cdc48 functions between Ub ligases and the proteasome, the only data that address the specific targeting step on which Cdc48 acts to promote turnover of soluble proteins are those underlying the “escort” model (Richly et al., 2005). This model posits that Cdc48 takes over from Ub ligases by coordinating the elongation of a size-restricted yet degradation-competent chain upon substrate, whereupon the substrate is handed off to an Ub chain receptor such as Rad23 for delivery to the proteasome. Sculpting of the Ub chain is achieved by a combination of trimming by deubiquitinating enzymes (DUBs) such as Otu1 and chain

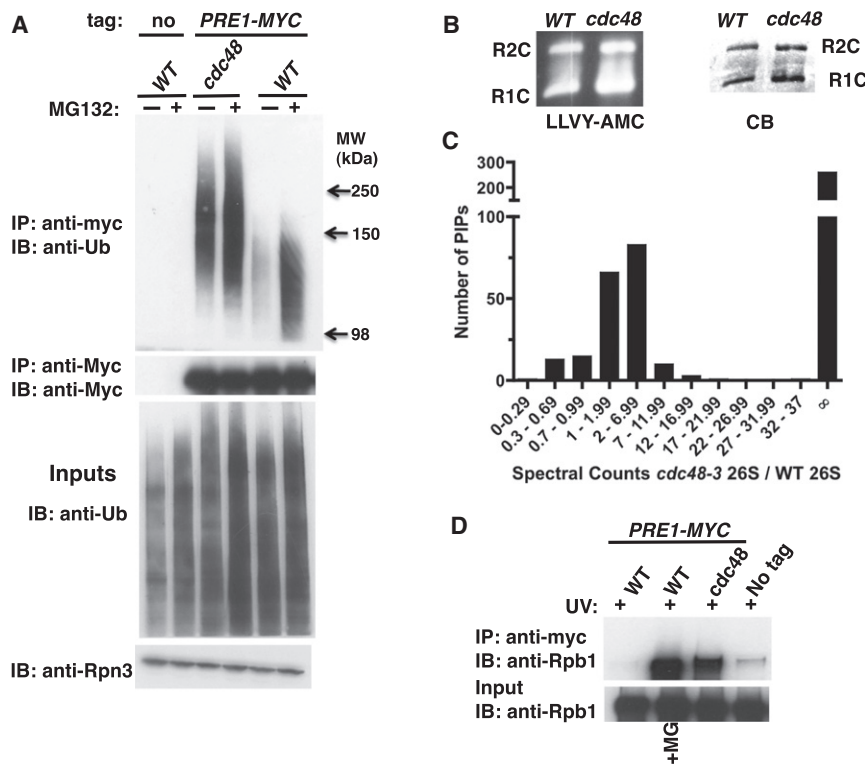


Figure 1. Ubiquitinated Proteins Are Targeted to 26S Proteasomes in *cdc48-3* Mutant Cells

(A) Ub conjugates accumulated on proteasomes in *cdc48-3*. Wild-type (WT; RJD3437) and mutant (RJD3454) cells expressing myc-tagged Pre1 (or not, RJD4090) were shifted to 37°C for 90 min and treated with 40 μM MG132 for 30 min. Proteasomes were isolated by anti-myc immunoprecipitation (IP) and immunoblotted (IB) for Ub and Pre1-myc (upper panels). Input extracts were blotted for Ub and Rpn3 (lower panels).

(B) Accumulation of conjugates on proteasome was not due to a defect in chymotryptic activity in *cdc48-3* mutants. Isolated 26S proteasomes fractionated by native PAGE were either stained with Coomassie blue (CB) or processed for in-gel peptidase activity using the fluorescent reporter substrate LLVY-AMC. R1C and R2C refer to 20S complexes capped by one or two 19S regulatory particles, respectively.

(C) Numerous proteins accumulated on proteasomes in *cdc48-3* mutants. Multidimensional mass spectrometry (MudPIT) was performed on an LTQ mass spectrometer with 26S proteasomes isolated from WT and mutant (RJD2902) cells grown at 37°C for 2 hr. The actual ratio of total spectral counts of proteasomal subunits from mutant to WT was 0.8 and was normalized to one. The same normalization factor was applied for spectral counts obtained for the proteasome-interacting proteins (PIPs). ∞ refers to proteins found in *cdc48-3* but not WT proteasomes.

(D) Rpb1 accumulated on proteasome in *cdc48-3* mutant treated with UV. WT and mutant cells were shifted to 37°C for 90 min and UV irradiated. Cells were recovered at 37°C for 30 min in the absence or presence of MG132. Input cell extracts and proteasomes immunoprecipitated (IP) with anti-myc were blotted (IB) for Rpb1 using 4H8 antibody.

See also Figure S1.

extension by the “E4” enzyme Ufd2 (Rumpf and Jentsch, 2006). A central prediction of the escort model is that proteasome-bound Ub conjugates should become depleted in Cdc48 mutants, much as is seen in *rad23Δdsk2Δ* double mutants (Elsasser et al., 2004), but this has not been evaluated.

Here, we characterize 26S proteasomes isolated from wild-type and temperature-sensitive conditional *cdc48-3* mutant cells. Contrary to expectation, Ub conjugates and many nonproteasomal proteins accumulated to high levels on proteasomes from *cdc48-3* mutants. In-depth analysis of one particular Cdc48-dependent turnover substrate, Rpb1, reveals that Cdc48 and its adaptor Ubx5 function downstream of Cul3 Ub ligase to facilitate degradation of chromatin-bound Rpb1.

RESULTS

Ub Conjugates and Numerous Proteins Accumulate on Proteasomes Isolated from *cdc48-3* Mutants

To determine whether Ub conjugates are targeted to proteasomes in the absence of Cdc48 function, we isolated 26S proteasomes from wild-type and *cdc48-3* cells. Cdc48 was not required for proteasome assembly (Figure S1A available online). We next evaluated Ub by immunoblotting and surprisingly observed that proteasomes purified from *cdc48-3* cells contained an increased level of Ub conjugates compared to the

wild-type (Figure 1A). Indeed, the level of conjugates associated with proteasomes isolated from mutant cells was comparable to that observed with proteasomes purified from wild-type cells treated with the proteasome inhibitor MG132. Additionally, the conjugates were of unusually high molecular weight (HMW; 110 kDa and greater). One explanation for this result is that Cdc48 was required for proteasomal peptidase activity, but this was ruled out by an in-gel peptidase assay on 26S proteasomes fractionated by native PAGE (Figure 1B).

The temperature-sensitive *cdc48-3* allele bears two point mutations, P257L and R387K (Jeffrey Laney, personal communication). The molecular effects of these mutations are not known, but the mutant protein is stable at the restrictive temperature (data not shown). To assess more directly whether the ATPase activity of Cdc48 contributed to degradation of proteasome-bound Ub conjugates, we transformed mutant cells with plasmids that expressed either wild-type or ATPase-dead (pcdc48Q2) Cdc48 (Ye et al., 2003). Ub conjugate accumulation on 26S proteasomes isolated from *cdc48-3* was suppressed by coexpression of wild-type Cdc48 but was further enhanced in cells expressing the ATPase-deficient Cdc48 (Figure S1B).

To compare the protein composition of 26S proteasome complexes affinity-purified from wild-type and *cdc48-3* cells, we subjected the preparations to multidimensional mass spectrometry (MudPIT) (Graumann et al., 2004; Mayor et al., 2005)

with an LTQ (Figure 1C and Figure S1D) or LCQ (Figure S1C) mass spectrometer and measured spectrum counts, which correlate with protein abundance (Weiss et al., 2010). The ratio of total proteasomal subunit spectral counts from mutant to wild-type was close to 1, indicating consistent recovery of equivalent amounts of proteasome from wild-type and *cdc48-3* cells. Moreover, the spectrum counts observed for individual subunits (Figure S1D) were similar for both preparations. We next determined the spectral counts for proteasome-interacting proteins (PIPs) in each preparation and calculated the ratio as above. Notably, and in striking contrast to proteasome subunits, spectrum counts for most PIPs were higher in *cdc48-3* proteasomes, and a large number of PIPs were found only in the mutant preparation (Figure 1C and Figure S1C).

We reasoned that proteins found at elevated levels in proteasomes isolated from *cdc48-3* cells might be substrates that could not be degraded due to incomplete unfolding or extraction from binding partners. To identify candidate substrates, we mined data from four data sets: (1) all proteins in yeast with a half-life of less than 55 min (Belle et al., 2006), (2) proteins reported to be ubiquitinated in a large-scale proteomic study (Peng et al., 2003), (3) proteins that accumulate as Ub conjugates upon UPS inhibition (Mayor et al., 2007), and (4) proteins reported in the *Saccharomyces* Genome Database (SGD, <http://www.yeastgenome.org/>) to have physical or genetic links with Ub ligases. Several PIPs were found to overlap between two data sets, an example being the septin-localized checkpoint kinase Hsl1, which is degraded via APC/C in G1 (Burton and Solomon, 2000). However, one candidate—the largest subunit of RNA polymerase II, Rpb1—was identified in all four data sets. Although we chose to focus the remaining study on Rpb1, we did confirm that Hsl1 degradation was dependent on Cdc48 (Figure S2).

Rpb1 is ubiquitinated in the absence of any inducing signal (Daulny et al., 2008; Peng et al., 2003) and also upon stalling of transcription by drugs such as 6-azauracil (6-AU) that deplete intracellular nucleotide pools (Somesh et al., 2007). These data suggest that there may be basal turnover of Rpb1 fueled by stalling of transcription throughout the genome (Sigurdsson et al., 2010). However, upon induction of DNA damage by ultraviolet radiation (UV) or by the UV-mimetic 4-nitroquinoline-1-oxide (4-NQO), there is a large induction of Rpb1 turnover (Beaudenon et al., 1999; Chen et al., 2007; Ribar et al., 2006, 2007; Somesh et al., 2005). To evaluate whether Rpb1 accumulates on proteasomes in UV-treated *cdc48-3* cells, we irradiated wild-type (\pm MG132) and *cdc48-3* cells with UV, isolated proteasomes, and immunoblotted for Rpb1. As shown in Figure 1D, MG132 induced accumulation of Rpb1 on proteasomes from UV-irradiated wild-type cells. Strikingly, there was also a strong accumulation of Rpb1 on proteasomes isolated from UV-irradiated *cdc48-3* cells.

UV-Induced Degradation of Rpb1 Is Dependent on Cdc48

To address whether Cdc48 is involved in the degradation of Rpb1, we treated wild-type and *cdc48-3* cells with UV and allowed them to recover in the presence of cycloheximide to block protein synthesis. Aliquots were collected at various time

intervals, and Rpb1 levels were monitored by immunoblotting. Rpb1 was degraded upon UV irradiation of wild-type but not *cdc48-3* mutant cells (Figure 2A). Quantification of the immunoblot and normalization with tubulin yielded data similar to Figure 2C (data not shown). A similar stabilization was observed when Rpb1 turnover was induced by 4-NQO, and Rpb1 accumulated on 26S proteasomes isolated from 4-NQO-treated *cdc48-3* cells to levels comparable to wild-type cells treated simultaneously with 4NQO and MG132 (Figures S3C and S3D). Stabilization of Rpb1 in UV-treated *cdc48-3* was not due to arrest of this mutant at the mitosis checkpoint (Cheng and Chen, 2010), because we also observed stabilization in checkpoint-deficient *cdc48-3mad2 Δ* mutants (Figure S3A) and in *cdc48-3* at a temperature (30°C) that is permissive for mitosis (Figure S3B). To determine whether the ATPase activity of Cdc48 was required for Rpb1 degradation, we analyzed *cdc48-3* mutants transformed with the same plasmids used for the experiment in Figure S1B as described above. Whereas wild-type *CDC48* rescued the degradation defect of the *cdc48-3* allele (the only allele tested), the ATPase mutant did not (Figure 2B).

Prior work has shown that Rpb1 ubiquitination and degradation is compromised in UV-irradiated *def1 Δ* cells. Surprisingly, Def1's function in Rpb1 degradation can be fully bypassed if *RAD26* is deleted, suggesting that Rad26, which is a DNA-dependent ATPase homologous to mammalian Cockayne Syndrome B (CSB), prevents access to the ubiquitination machinery (Woudstra et al., 2002). We wished to determine whether Cdc48 might function indirectly to overcome a *RAD26*-dependent barrier to Rpb1 degradation. Whereas Rpb1 degradation was accelerated in *rad26 Δ* cells, the *cdc48-3 rad26 Δ* double mutant was as compromised for degradation as *cdc48-3* alone (Figure 2C). Given that Cdc48 remained essential for Rpb1 turnover in *rad26 Δ* cells, we reasoned that its role might be direct. To assess this, we performed a coimmunoprecipitation experiment (Figure 2D). Rpb1 was specifically coprecipitated with Cdc48, but their interaction was UV independent. This observation suggests an additional, yet-to-be-discovered role for Cdc48 in Rpb1 transactions independent of UV damage (see below).

Dependency of Rpb1 Degradation on Cdc48 Adaptor Proteins

Cdc48 is a mechanochemical transducer that is coupled to its ubiquitinated substrates by adaptor proteins. As shown in Figure 3A, UV-dependent turnover of Rpb1 was also dependent on the Ufd1-Npl4 adaptor complex. Cdc48/p97 also interacts with a second set of putative substrate receptors, the UBX domain proteins. Shp1/Ubx1 and its mammalian homolog p47 are believed to be substrate recruitment factors for Cdc48/p97 that are mutually exclusive with Ufd1-Npl4 (Table S2), and Ubx1 is required for the degradation of UFD pathway reporter substrates in budding yeast (Schuberth et al., 2004). We evaluated UV-induced Rpb1 turnover in mutants individually lacking each one of the UBX proteins and found that Rpb1 was stabilized in *ubx1 Δ* , *ubx5 Δ* , and *ubx4 Δ* , whereas degradation was unimpeded in *ubx2 Δ* , *ubx3 Δ* , *ubx6 Δ* , and *ubx7 Δ* mutants (Figures 3B and 3C). As will be shown below, the defect in *ubx1 Δ* was due to an indirect effect on UV signaling, and thus we focused

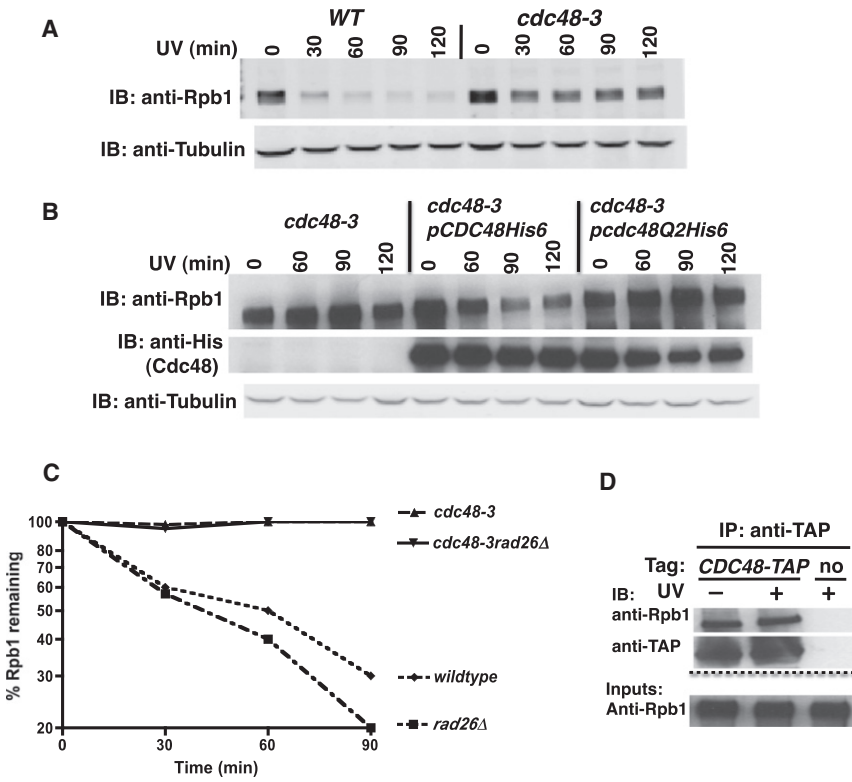


Figure 2. The ATPase Activity of Cdc48 Is Required for UV-Induced Turnover of the Largest Subunit of RNA Polymerase II, Rpb1

(A) Rpb1 was stabilized in *cdc48-3* cells irradiated with UV. WT (RJD360) and mutant (RJD3411) cells were shifted to 37°C for 1 hr, irradiated with UV, and transferred to medium containing 100 μg/ml cycloheximide. At the indicated time points, cells were harvested and lysed for immunoblot (IB) analysis of Rpb1 using 8WG antibody. Tubulin served as the loading control.

(B) WT, but not ATPase-deficient Cdc48, restored Rpb1 turnover in UV-irradiated *cdc48-3* cells. Mutant *cdc48-3* (RJD3411) cells containing plasmid-borne WT *GAL-CDC48His6* (RJD4996) or the Q2 mutant (RJD4997) were grown in galactose for 2 hr to induce expression of ectopic Cdc48. Induced cells were shifted to 35°C (the minimum restrictive temperature in this medium) for 1 hr, UV irradiated, and then sampled at the indicated time points.

(C) Stabilization of Rpb1 in *cdc48-3* was not suppressed by *rad26Δ*. Single (RJD3411 and 4523) and double (RJD4570) mutants were shifted to 37°C for 1 hr, UV irradiated, and processed as above. Quantification was performed on LI-COR Odyssey with normalization to tubulin. After quantification, all data were plotted with Prism software on a logarithmic scale on the y axis.

(D) Cdc48 associated with Rpb1. Cells were UV treated or not and lysates prepared for immunoprecipitation (IP). Aliquots of inputs and immunoprecipitates were analyzed for their content of Cdc48 and Rpb1 by blotting (IB) with TAP and 4H8 antibodies, respectively. See also Figure S3.

our effort on Ubx4 and Ubx5. To determine whether these proteins function in the same or parallel pathways, we quantified UV-induced Rpb1 turnover in single and double mutants. Rpb1

was more stable in *ubx4Δ ubx5Δ* than in either single mutant, suggesting that these proteins act in parallel to promote Rpb1 degradation (Figure 3D).

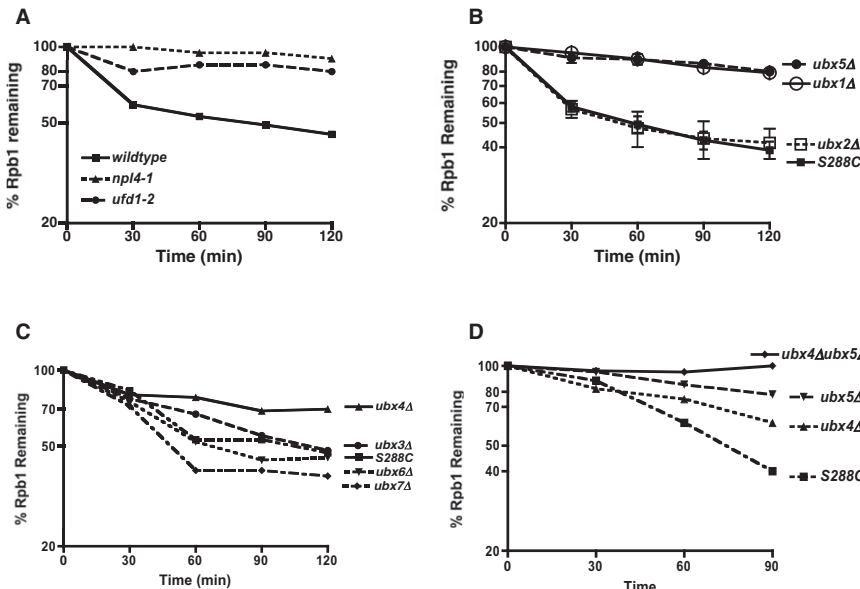


Figure 3. Multiple Cdc48 Adaptors Are Involved in Rpb1 Degradation

(A) Npl4 and Ufd1 contributed to Rpb1 turnover. Wild-type and mutant cells (*npl4-1*/RJD2589 maintained at 30°C, and *ufd1-2*/RJD3264 shifted to 37°C for 1 hr) were UV irradiated at 0 min. Cultures were processed as described in Figure 2C.

(B) Ubx1 and Ubx5 contributed to Rpb1 turnover. WT (S288C) and mutant cells (RJD4614, RJD2551, RJD3176, RJD3177) maintained at 30°C were irradiated with UV at 0 min. Cultures were processed as described in Figure 2C. Results are presented as means ± SEM of three independent experiments.

(C) Ubx4 contributed to Rpb1 turnover. WT and mutant cells (RJD5249, RJD5246, RJD5247, RJD5248) maintained at 30°C were irradiated with UV at 0 min. Cultures were processed as described in Figure 2C.

(D) Increased impairment of Rpb1 turnover in *ubx4Δubx5Δ* double mutants compared to single mutants. WT and mutant cells (RJD5259) maintained at 30°C were irradiated with UV at 0 min. Cultures were processed as described in Figure 2C.

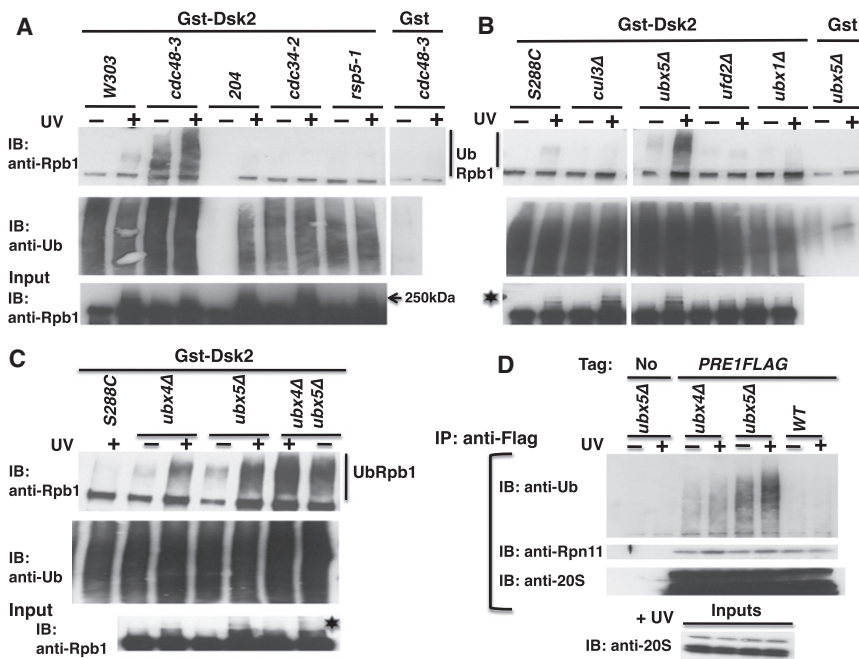


Figure 4. Rpb1 Accumulates in the Ubiquitinated State in *cdc48-3*, *ubx4Δ*, *ubx5Δ*, and *ubx4Δubx5Δ* Mutants

(A) WT (*W303* and *204*) and mutant cells were shifted to 37°C for 90 min and UV irradiated. Lysates were fractionated on a GstDsk2 resin. The input extract and bound fractions were immunoblotted (IB) for Rpb1 (4H8) and Ub, as indicated. *204* is RJD487, a wild-type congenic to *cdc43-2*, and untreated sample and beads were partially lost during IP.

(B) Rpb1 Ub conjugates accumulated in *ubx5Δ* but not *ubx1Δ*, and were diminished in *cul3Δ* mutant cells. Methods were the same as in (A) except that cells were maintained at 30°C. The asterisk indicates sumoylated Rpb1.

(C) Rpb1 Ub conjugates hyperaccumulated in a *ubx4Δubx5Δ* double mutant. Methods were the same as in (A) except that cells were maintained at 30°C.

(D) Ub conjugates accumulated on proteasomes isolated from *ubx4Δ* and *ubx5Δ* mutants.

Lysates from wild-type and mutant cells expressing Flag-tagged Pre1 (or not) were immunoprecipitated (IP) with anti-Flag and immunoblotted (IB) for Ub, Rpn11, and 20S. Input extracts were blotted for 20S.

See also Figure S5.

Ongoing transcription is a prerequisite for UV-induced RNA Pol II degradation (Anindya et al., 2007). To evaluate the possibility that the UBX proteins were involved in global transcription, we determined the sensitivity of the adaptor null mutants to the nucleotide-depleting drug 6-AU, which affects both elongation rate and processivity of RNA Pol II (Mason and Struhl, 2005). Whereas a null mutant lacking Dst1/TFIIS, a general transcription elongation factor, was 6-AU sensitive, the *ubxΔ* mutants were 6-AU insensitive (Figure S4A). Additionally, because Rpb1 degradation was significantly impaired in *cdc48-3* mutants even at the semirestrictive temperature 30°C (Figure S3B), we evaluated 6-AU, and UV sensitivity of *cdc48-3* at 30°C. The sensitivities of the mutant were akin to the wild-type (Figures S4A and S4B). Thus, transcription elongation and UV signaling were intact in *cdc48-3* cells under conditions where UV-induced degradation of Rpb1 was severely compromised.

Ubiquitinated Rpb1 Accumulates in *cdc48-3*, *ubx4Δ*, and *ubx5Δ* Cells

We have shown above that Cdc48 and three of its UBX domain adaptors were required for Rpb1 turnover. The dependency on Cdc48-Ubx could reflect a requirement for segregase (Braun et al., 2002) activity such that the Rpb1 degron(s) remains masked through interaction with its holoenzyme partners in *cdc48-3* or *ubxΔ* cells, blocking access to Ub ligases, or the dependency could reflect a requirement for segregase/unfolding activity after ubiquitination. To assess the modification status of Rpb1 in mutants, we immunoprecipitated Rpb1 from control and UV-irradiated wild-type cells and then blotted the sample with an Rpb1 antibody. A discrete, UV-stimulated modification of a small fraction of Rpb1 was reproducibly observed (Figure S5A). This modification was unaffected by *cdc48-3*, *ubx4Δ*, and *ubx5Δ*

mutations but was compromised in *ubx1Δ* mutants (see inputs/asterisk, Figure 4). Prior work established that this modification is due to sumoylation (Chen et al., 2009). We confirmed that Rpb1 was sumoylated in *cdc48-3* but not *ubx1Δ* cells after UV irradiation (Figure S5B) and that sumoylation did not affect Rpb1 degradation (Figure S5C). These data establish that the UV damage response remains intact in *cdc48-3*, *ubx4Δ*, and *ubx5Δ* but not *ubx1Δ* mutants.

Typically, when degradation of a protein is blocked downstream of ubiquitination (e.g., with MG132), the fraction that accumulates as Ub conjugates is very small (Liu et al., 2007). To enhance our ability to detect the HMW polyubiquitinated pool of Rpb1 (UbRpb1), we exploited the ability of the UBA domain of Dsk2 to bind polyUb conjugates (Mayor et al., 2005). Lysates from wild-type and mutant cells were bound to GstDsk2. As expected, GstDsk2 retrieved Ub conjugates but Gst did not (middle panels, Figures 4A–4C). Immunoblotting for Rpb1 revealed the accumulation of HMW conjugates in *cdc48-3* cells. No accumulation of Rpb1 Ub conjugates was observed in either the Ub-conjugating enzyme mutant *cdc34-2* or in the Ub ligase-deficient mutants *rsp5-1* and *cul3Δ*. Cul3 generates the K48-linked Ub conjugates on Rpb1 that signal its degradation. It has been suggested that Cul3 functions either alone (Ribar et al., 2007) or downstream of Rsp5 (Harreman et al., 2009).

Among the *ubxΔ* mutants, UbRpb1 was recovered from UV-treated *ubx4Δ* and *ubx5Δ* cells but not *ubx1Δ* cells. Moreover, accumulation of constitutively ubiquitinated Rpb1 was also detected in *cdc48-3*, *ubx4Δ*, and *ubx5Δ* (Figures 4A–4C), suggesting that Cdc48 and its adaptors act upon Rpb1 stalled at naturally occurring pause sites such as at the 5' end of open reading frames (ORFs) (Sigurdsson et al., 2010; Wade and

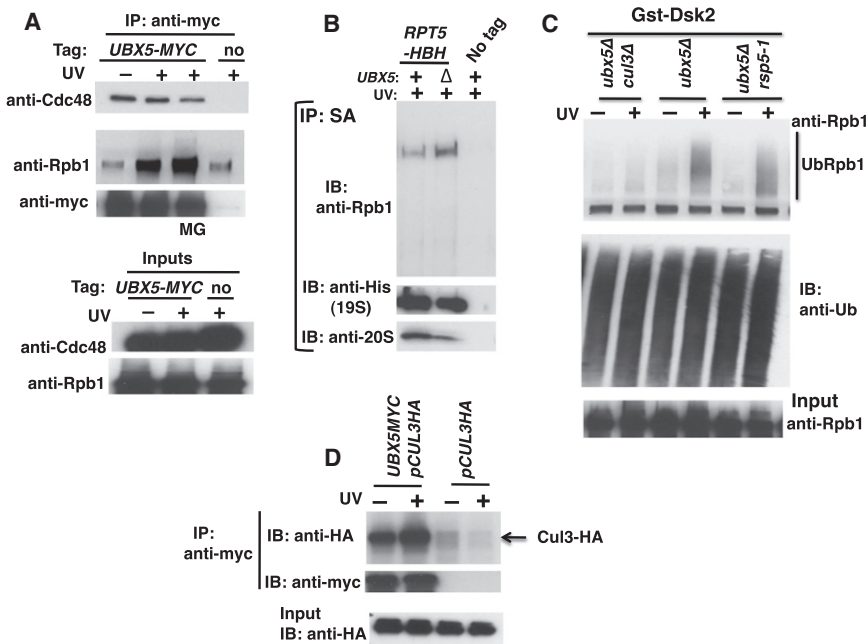


Figure 5. Ubx5-Cdc48 Acts Downstream of Cul3 in UV-Dependent Rpb1 Degradation

(A) UV irradiation stimulated interaction of Ubx5 with Rpb1. Untagged (RJD4614), and *UBX5-MYC* (RJD4214) strains were UV irradiated or not, and lysates were immunoprecipitated (IP) with anti-myc antibody. Aliquots were blotted (IB) for Cdc48 and Rpb1 (with 4H8). MG, +MG132.

(B) Rpb1 accumulated on 26S proteasomes isolated from *ubx5Δ* cells. Cells expressing HBH-tagged Rpt5 (RJD4741 and RJD4742) were irradiated with UV and crosslinked with formaldehyde. Lysates were prepared in 6M guanidine-HCl and 26S proteasomes isolated by consecutive Ni²⁺-NTA and streptavidin (SA) affinity chromatography under denaturing conditions. Purified samples were blotted (IB) for Rpb1 (4H8), Rpt5-HBH (anti-His), and 20S (anti-a7).

(C) Ub conjugates that accumulated in *ubx5Δ* cells were substantially dependent on Cul3 but not Rsp5. Methods were the same as in Figures 4A and 4B.

(D) Ubx5 and Cul3 interacted. Cells expressing HA-tagged Cul3 from a plasmid and Myc-tagged Ubx5 were irradiated with UV or not. Input extracts and anti-myc immunoprecipitates (IP) were analyzed for their content of Cul3 by immunoblotting (IB) with anti-HA.

Struhl, 2008) or at noncanonical DNA structures (Hanawalt and Spivak, 2008). Interestingly, accumulation of UbRpb1 was enhanced in *ubx4Δubx5Δ* double mutants compared to either single mutant (Figure 4C), providing further evidence that Ubx4 and Ubx5 function in parallel pathways. To determine whether *ubx4Δ* or *ubx5Δ* elicits a more global defect in proteolysis, proteasomes were isolated from wild-type and mutant cells and immunoblotted for Ub conjugates. Strikingly, loss of either adaptor resulted in accumulation of HMW conjugates (Figure 4D), as was observed upon thermal inactivation of Cdc48 function (Figure 1A).

Ubx5 Binds Rpb1 and Cul3 and Functions Downstream of Cul3

To investigate in greater detail how UBX protein function contributes to Rpb1 degradation, we focused our attention on Ubx5 because *ubx5Δ* exhibits a stronger phenotype than *ubx4Δ* in (1) Rpb1 stabilization, (2) UbRpb1 conjugate accumulation, and (3) Ub conjugate accumulation at the proteasome. Since Cdc48 bound Rpb1 (Figure 2D), we tested whether Ubx5 behaves likewise. Ubx5 bound Cdc48 constitutively, as expected, but its association with Rpb1 was strongly enhanced by UV irradiation (Figure 5A). This is an interesting counterpoint to Cdc48, which bound Rpb1 independently of DNA damage (Figure 2D). These observations suggest that Cdc48 engages in multiple transactions with Rpb1, with different transactions mediated by different adaptors. Additionally, as observed for *cdc48-3* mutants, Rpb1 accumulation was enhanced on 26S proteasomes isolated under denaturing conditions from formaldehyde-crosslinked *ubx5Δ* cells (Figure 5B).

As noted above, both Rsp5 and Cul3 have been implicated in Rpb1 degradation. To test whether Ubx5 functioned down-

stream of one or both of these Ub ligases, we evaluated UbRpb1 levels in *ubx5Δ* mutants lacking one or the other enzyme. Interestingly, *cul3Δ* but not *rsp5-1* severely attenuated recovery of UbRpb1 from *ubx5Δ* cells (Figure 5C). Given that Cul3 and Ubx5 could be coimmunoprecipitated (Figure 5D), we suggest that these proteins normally act sequentially, such that Rpb1 that has been ubiquitinated by Cul3 is “handed off” to Ubx5. These data extend observations made in human cells, where UBXD7 is linked to the CUL2-VHL complex and its substrate HIF1 α (Alexandru et al., 2008). Bioinformatic analysis suggests that Ubx5 is the ortholog of UBXD7 (Schuberth and Buchberger, 2008).

Accumulation of Ubiquitinated Rpb1, Cdc48, and Proteasome on Chromatin

The results presented thus far suggest that Ubx5-Cdc48 acts on Rpb1, downstream of Cul3, to mediate degradation of ubiquitinated Rpb1 formed upon UV irradiation. The question that arises next is whether this sequence of events occurs on chromatin. If so, we would expect to detect the core components of the system—including Cul3, Cdc48, and the proteasome—on chromatin in UV-treated cells. To address this question, we employed a standard chromatin fractionation method (Liang and Stillman, 1997). Interestingly, the bulk of Cul3 fractionated with chromatin (Figure 6A). Cul3 was constitutively associated with chromatin, which is not surprising given that this enzyme is likely to have multiple substrates. To stringently probe the association of the abundant Cdc48 and proteasome complexes with chromatin, we subjected a crude chromatin pellet to limited micrococcal nuclease (MNase) digestion followed by centrifugation to yield a highly purified chromatin pellet consisting of polynucleosomes (Frc6) and the supernatant (Frc7). Frc6, which provides a stringent assessment of chromatin association (Liang

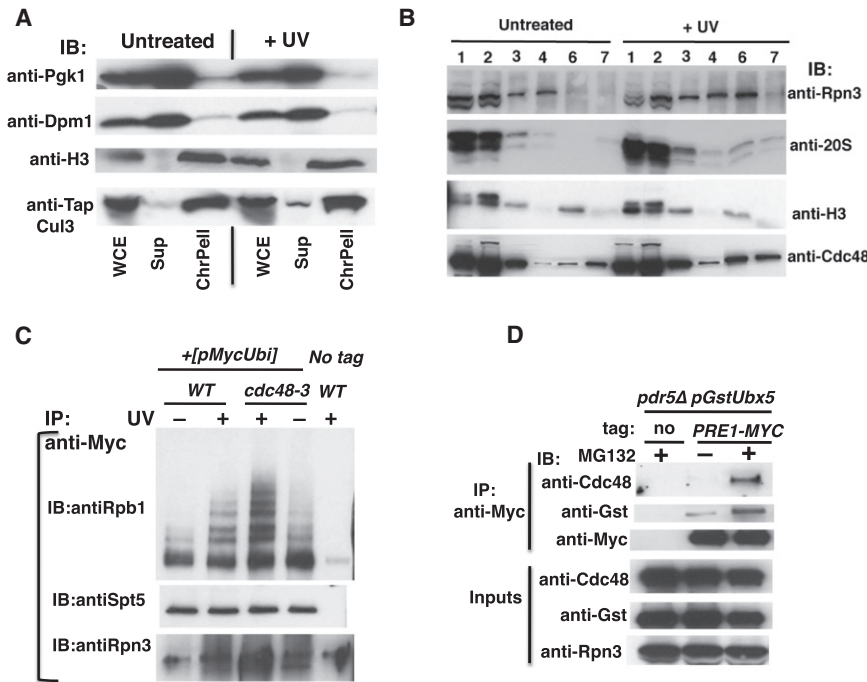


Figure 6. Ubiquitinated Rpb1 Accumulates on Chromatin in UV-Treated *cdc48-3* Mutant

(A) *CUL3-TAP* (RJD4680) cells were treated with UV or not and harvested after the addition of 0.1% azide. Spheroplasts were prepared with 25 units of Zymolyase 100T for 2×10^9 cells. The three fractions, whole-cell extract (WCE), supernatant (Sup), and chromatin pellet (ChrPel), derived from spheroplasts were immunoblotted (IB) with the indicated antibodies.

(B) Cdc48 and 26S proteasome recruitment to chromatin was enhanced after UV. Crude chromatin pellets (Frc3) obtained by centrifuging WCE (Frc1) through a sucrose cushion were fractionated further after limited digestion with micrococcal nuclease (MNase) to generate high-speed pellet (Frc6) and supernatant (Frc7) fractions. Frc6 is enriched in released polynucleosomes. All fractions were immunoblotted (IB) for histone H3, 19S (Rpn3), 20S proteasome subunits, and Cdc48. Fraction numbers correspond to lane numbers.

(C) Ubiquitinated Rpb1 accumulated on chromatin in response to UV and inactivation of Cdc48. WT and mutant cells expressing Myc-tagged Ub were shifted to 37°C, and Cu²⁺ was added to induce expression of Ub. After 2 hr, cultures were irradiated with UV (or not) and harvested.

Crude chromatin pellets (Frc) were isolated as in (B) and treated with Benzonase. Solubilized material was immunoprecipitated (IP) with anti-myc, and aliquots were blotted (IB) for Rpb1 (4H8), Spt5, and Rpn3.

(D) Cells of the indicated genotype were galactose-induced for 2 hr, then treated (or not) with MG132 for 30 min, lysed, and subjected to immunoprecipitation with anti-myc. The resulting samples were immunoblotted with antibodies to detect the indicated antigens. pGST-UBX5 is a plasmid that expresses GST-tagged Ubx5 under the *GAL* promoter.

See also Figure S6.

and Stillman, 1997), was enriched for Cdc48 as well as both 19S and 20S proteasome components after UV irradiation, whereas Histone H3 was constitutively present and served as a loading control (Figure 6B). Given that both Cdc48 and the proteasome are likely to have many substrates, our data suggest that UV damage triggers a generalized response that may result in the removal or degradation of many chromatin proteins.

Based on the observation that Cul3, Cdc48, and the proteasome can all be found on chromatin in UV-stressed cells, we hypothesized that ubiquitination and degradation of Rpb1 occurs on chromatin. To test this idea, we examined chromatin-bound Rpb1. Crude chromatin (see Figures S6A and S6B for fractionation and loading controls) isolated from cells that expressed Myc-tagged Ub was solubilized with Benzonase and ubiquitinated chromatin proteins released into the supernatant were isolated by virtue of the Myc tag. Immunoblotting for Rpb1 revealed the presence of Ub ladders on chromatin-associated Rpb1 (Figure 6C). Importantly, the abundance of chromatin-bound Rpb1 Ub conjugates was greatly increased upon UV irradiation of wild-type cells, and increased to even higher levels in UV-irradiated *cdc48-3* cells. Thus, it appears that Rpb1 is ubiquitinated on chromatin, consistent with the idea that the degradation cascade initiates with ubiquitination of Rpb1 arrested at a thymidine dimer.

Since the recruitment of both Cdc48 and the 26S proteasome to chromatin was enhanced after UV, we wished to determine

whether both complexes could be coimmunoprecipitated. Earlier work had relied on *in vivo* crosslinking to detect binding of Cdc48 to the proteasome (Guerrero et al., 2008). Immunoprecipitation of 26S proteasome under native conditions in the presence of ATP and 0.15 M salt resulted in barely-detectable recovery of Cdc48 (Figure 6D), although Ubx5 could be readily detected after mild overproduction. Treating cells with the proteasome inhibitor MG132 enhanced interaction of both Cdc48 and Ubx5 with the proteasome, suggesting that binding was mediated in part by ubiquitinated substrates. This is consistent with the data in Figure 1A and supports the idea that Cdc48 can act upon substrates bound to the proteasome to enable their degradation.

DISCUSSION

Implicit in the models for Cdc48/p97 function based on its well-documented role in retrotranslocation of ubiquitinated proteins from the ER into the cytosol (Hirsch et al., 2009; Raasi and Wolf, 2007) and in the degradation of Ub fusion proteins (Richly et al., 2005) is the notion that it functions upstream of the proteasome shuttle receptors Rad23 and Dsk2. These models imply that Cdc48 is required for delivery of a subset of UPS substrates (i.e., Cdc48/p97-dependent substrates) to the proteasome. Our data in the current study demonstrating accumulation of Ub conjugates and PIPs on proteasomes isolated from *cdc48-3* mutants challenge whether these models serve as a general

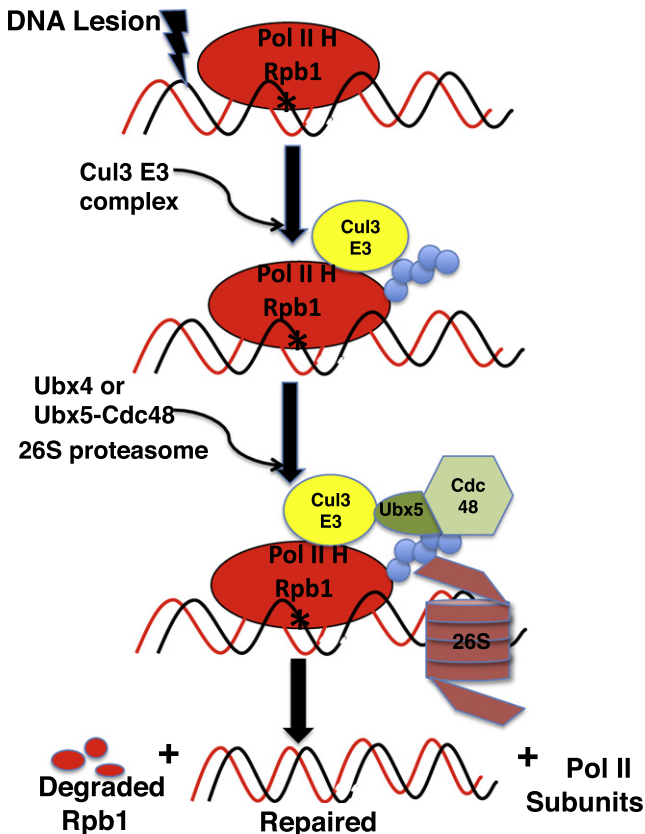


Figure 7. Hypothetical Model for Damage-Dependent Turnover of Rpb1

The Rpb1 subunit of RNA Pol II holoenzyme (H) irreversibly stalled at sites of DNA lesions is ubiquitinated by the Cul3-RING ligase complex. UbRpb1 can independently recruit proteasome and Ubx5-Cdc48 complexes. UbRpb1 is extracted from its binding partners in an unfolding reaction dependent on Ubx4 or Ubx5-Cdc48 and is threaded into the 26S proteasome.

paradigm for the temporal staging of Cdc48 function. Here, we validated Rpb1 as a bona fide substrate of Cdc48 and show that in cells in which Rpb1 degradation was induced by UV radiation, Rpb1 was delivered to the proteasome in the absence of Cdc48 function.

The Pathway for Degradation of Rpb1 in UV-Treated Cells

Our model for Rpb1 degradation (Figure 7) is that Rpb1 stalled at lesion sites is most often dislocated from the damage to enable repair followed by resumption of transcription. However, Rpb1 that is persistently blocked or refractory to dislocation is eventually modified by the Ub ligases Rsp5 and Cul3, of which the latter is believed to decorate Rpb1 with degradation-competent Ub chains. These chains enable recruitment of both Cdc48 and 26S proteasomes to the stalled complex, resulting in degradation of Rpb1 and recycling of the other holoenzyme subunits, which are not degraded (Malik et al., 2008). We envision that Cdc48 mediates extraction of UbRpb1 from chromatin-bound Pol II holoenzyme and that this extraction and subsequent Rpb1 degradation are tightly coupled, possibly even contempo-

aneous. Cdc48 carries out its function in conjunction with Ufd1-Npl4, Ubx4, and Ubx5. We do not know how the activities of Ufd1-Npl4 relate to those of the Ubx proteins. On the other hand, Ubx4 and Ubx5 appear to act in parallel pathways to promote Rpb1 degradation.

It has been proposed that Rsp5 attaches an initiator Ub to Rpb1, which primes polymerization of an Ub chain by Cul3 (Harreman et al., 2009). Alternatively, it has been suggested that Cul3 carries out the entire reaction and the role of Rsp5 is indirect (Ribar et al., 2007). Ubiquitinated Rpb1 that accumulated in *ubx5Δ* cells was greatly diminished by deletion of *CUL3* but barely affected by *rsp5-1*. Either Rsp5 is not required to generate the UbRpb1 that accumulates in *ubx5Δ*, or the *rsp5-1* mutation was leaky under our conditions. Regardless of whether or not Cul3 extends Ub chains initiated by Rsp5, the epistasis data and the protein-protein interactions point to Ubx5 acting directly upon ubiquitinated Rpb1 formed by Cul3.

Components involved in the Rpb1 turnover pathway have been linked with chromatin regulation in other contexts. Cul3 functions as part of an Ub ligase that ubiquitinates the UV-damage sensor Rad4 (XPC) (Gillette et al., 2006; Ramsey et al., 2004). Cdc48 promotes dissociation of a repressor from its target promoter (Wilcox and Laney, 2009), and p97 extracts protein kinase Aurora B from chromatin during nuclear envelope reassembly (Ramadan et al., 2007). Similarly, 19S and 20S subunits of the proteasome have been shown to bind promoters, ORFs, and termination regions, both constitutively and in response to signals such as HO endonuclease-induced double strand breaks and UV (Auld et al., 2006; Collins and Tansey, 2006; Gillette et al., 2004; Krogan et al., 2004). These data suggest that extraction of proteins from chromatin by Cdc48/p97—either coupled to proteasomal degradation or not—is likely to be a recurrent theme in chromatin regulation.

Proteasomal ATPases Are Insufficient for Segregation and Unfolding of Unstable Subunits of Some Macromolecular Complexes: Implications for Dependency on Cdc48

An unexpected insight from our work is that in cells deprived of Ubx5-Cdc48 function, substrates such as Rpb1 can gain access to the proteasome. This raises the question of why substrates accumulate on the proteasome in these cells. We suggest that extraction of UbRpb1 from chromatin-bound holoenzyme and targeting of UbRpb1 to the proteasome are normally coupled processes that do not occur obligately in a fixed order. Thus, depending upon the relative rates of disassembly versus targeting, in some instances the action of Cdc48 may precede substrate association with the proteasome (but this may be undesirable as explained below), whereas in other cases the substrate may associate with proteasome before Cdc48 can act. We suggest that the latter occurs far more commonly because disassembly/unfolding is likely to be much slower than targeting. For some substrates, like Rpb1 and the checkpoint kinase Hs1, the disassembling and/or unfolding activity contributed by the resident proteasomal ATPases (Rpt1-6) (Finley, 2009) may be insufficient to extract them from the macromolecular complexes in which they reside, and thus they must wait for Cdc48 to complete its job before their degradation can

commence. Coupling Cdc48 function to substrate degradation at the proteasome surface would reduce the probability that substrate that has been disassembled/unfolded by Cdc48 has an opportunity to reassemble, refold, or aggregate.

What is the molecular basis for determining whether or not a particular substrate requires Cdc48 for degradation? Prior work has established that stable domains within proteins are unfolded sequentially starting from the attachment point of the degradation signal (Lee et al., 2001; Prakash et al., 2004; Schrader et al., 2009). Rpb1 bound to multiple interacting proteins in the Pol II holoenzyme complex may lack an unstructured initiation site and thus may additionally require Cdc48 for preprocessing, as recently reported for stably folded UPS reporter substrates (Beskow et al., 2009). In stark contrast, the unfolding power of the 26S proteasome is sufficient to degrade to completion ubiquitinated Sic1 (UbSic1), even when it is tightly bound to S phase cyclin-dependent kinase (Verma et al., 2001). A potentially distinguishing feature of Sic1 is that it contains an intrinsically disordered N-terminal domain that mediates interaction with Ub ligase SCF (Mittag et al., 2008) and contains the primary sites of ubiquitination (Petroski and Deshaies, 2003). We speculate that because unraveling of UbSic1 initiates from the disordered region proximal to the Ub chain attachment site, the proteasome can complete the task without assistance. By contrast, we predict that Cdc48/p97 and its adaptors are required to sustain disassembly, unfolding, and processive degradation of soluble 26S proteasome substrates whose structural complexity overwhelms the proteasomal ATPases.

EXPERIMENTAL PROCEDURES

Turnover Analysis of UPS substrates

Aliquots of cultures treated as described in the respective figure legends (final A_{600} between 1 and 2) were collected and drop frozen in liquid nitrogen. Frozen cell pellets were thawed and washed with ice-cold Buffer A (50 mM Tris [pH 7.5], 10 mM sodium azide, 10 mM EDTA, 10 mM EGTA, 1 × protease inhibitor tablet [Roche], 10 mM NEM, 50 mM NaF, 60 mM β -glycerophosphate, and 10 mM sodium pyrophosphate). The cell pellets were then immersed in boiling water for 3 min, after which they were suspended in 1 × SDS buffer (37.5 μ l /optical density [O.D.] unit). An equal volume of glass beads (Sigma, 425–600 μ m, acid washed) was added, and cells were lysed by vortexing in Fast Prep-24 (MP) for 45 s at a setting of 6.5 and boiled again for 4 min. Boiled lysates were centrifuged at 16,000 × g for 1 min. Aliquots were resolved by SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S to determine equivalent loading of protein extracts. The nitrocellulose filters were immunoblotted with desired antibody and developed by ECL or quantified by LI-COR Odyssey with IR dye-linked secondary antibodies (Invitrogen). Anti-PSTAIRE (Santa Cruz), and anti-tubulin (Sigma) served as loading controls. After quantification, all data were plotted with Prism software on a logarithmic scale on the y axis. Anti-Rpb1 (clones 8WG, 4H8), anti-Myc, and anti-HA antibodies were from Covance.

UV and 4-NQO Treatment of Yeast Cells

Overnight cultures were diluted to an O.D. of around 0.2. When cells reached an O.D. of around 1, they were centrifuged and resuspended in 80% of the original culture volume in 2% Dextrose (or the sugar being used for the experiment). All of the following steps were carried out in the dark, under red safe light. Cultures were exposed to 400 J/m² of UV irradiation in either Petri dishes or glass trays, depending on volume. Irradiation was performed by using calibrated germicidal lamps (254 nm UV lamp, UVP, model number XX-405) or a prewarmed and calibrated Stratalink unit (Stratagene, model 2400). The UV meter used for calibration was purchased from UVP (Upland, CA; model number J225).

Cells were harvested immediately after UV or, when recovery was monitored, the 2% dextrose suspension was diluted into 20% volume of 5X prewarmed media containing 100 μ g/ml cycloheximide, and outgrowth continued in the dark. For 4-NQO treatment, exponential cultures were treated with the desired concentration from a 10 mg/ml stock stored in the dark.

Isolation of Chromatin Fraction

Essentially, the method of Liang and Stillman (1997) was followed with some modifications. In the original protocol, chromatin was isolated from yeast spheroplasts, and this method was followed in Figure 6A. Because Rpb1 was considerably degraded during the process of spheroplasting (particularly in UV-treated cultures), the method was modified to isolate chromatin fractions from yeast cells ground with a mortar pestle chilled in liquid nitrogen as described earlier (Verma and Deshaies, 2005) for Figures 6B and 6C. The minimum culture volume that could be ground with easy recovery of cell powder was 350 ml. Ground powder was weighed, and 2 × (wt/volume) extraction buffer (EB) containing 50 mM HEPES (pH 7.5), 100 mM KCl, 0.25% Triton, 2.5 mM MgCl₂, 25 mM NEM, 50 mM β -glycerophosphate, 5 mM sodium pyrophosphate, 1 × protease inhibitor tablet +EDTA, and 0.5 mM AEBSF was added.

Unlysed cells were pelleted at 5000 rpm (all centrifugations in refrigerated Eppendorf model 5417R) for 1 min. Ubiquitin-aldehyde (1 μ M) was added to the supernatant and an aliquot (25% vol/vol) was saved as WCE (Frc1). Another 25% of lysate was underlayered with 50% volume of 30% sucrose and centrifuged at 16,400 rpm for 15 min. The pellet was saved as low speed pellet (Frc3), and the supernatant was designated “low-speed supernatant” (Frc2). The remaining 50% of lysate was underlayered with sucrose and centrifuged to generate a second set of fractions 2 and 3. Crude fraction 3 was washed with EB at 12,000 rpm for 8 min, and the pellet was resuspended in EB. Resuspended chromatin pellet (375 μ l) was prewarmed to 37°C for 3 min after supplementing with CaCl₂ (2 mM final) and digested with 2 μ l 1:10 MNase (50% glycerol stock solution; from Sigma, 204.3 units/mg protein) for another 3 min. Digestion was stopped by the addition of EGTA to 5 mM. A low-speed micrococcal nuclease-treated pellet (Frc4) was generated by centrifuging at 10,000 rpm for 2 min at 4°C. The supernatant was centrifuged at 50,000 rpm (Sorvall RC M120EX, rotor number RP100AT4) for 1 hr to generate the high-speed pellet (Frc6), and supernatant (Frc7). Limited nuclease digestion retains chromatin proteins in the high-speed pellet fraction.

Solubilization of Chromatin

Washed crude chromatin pellet (Frc) was resuspended in EB (750 μ l) and NaCl was added to yield a final salt concentration of 0.6 M. Ubiquitin aldehyde (1 μ M) and benzonase (1 μ l of 250 U/ μ l, Novagen) were added and the sample was incubated for 30 min on ice, sonicated for 15 s (Amplitude at 20%), and then centrifuged at 10,000 rpm for 2 min. The supernatant was used for immunoprecipitations.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at doi:10.1016/j.molcel.2010.12.017.

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