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Article

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

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Supplemental Experimental Procedures

Yeast Strains.

Strains used in this study are described in Supplemental Table S1. They are derivatives of either the wildtype strain RJD 360 (W303 background), or the wildtype strain RJD 1721/4614 (S288C background). All conditional strains were confirmed to be *ts* by growing them at their restrictive temperature for 4-5 days. Deletion strains (S288C) were acquired from Open Biosystems. Deletions were confirmed by PCR, and epitope-marked strains by immunoblotting (besides the usual auxotrophic marker selection).

Isolation of 26S Proteasomes and TAP-tagged Cdc48 and Adaptors for Mass Spectrometry.

26S proteasomes were purified for mass spectrometric analysis as described (Verma and Deshaies, 2005). Briefly, Flag-tagged Pre1 (20S subunit) containing strains were grown as large-scale cultures (3 L), and lysed by grinding cell pellets in liquid nitrogen. Lysates were bound to anti-Flag beads, washed, and eluted with Flag Peptide. TAP-tagged Cdc48 and its adaptors were purified by a tandem affinity protocol as described (Rigaut et al., 1999) and eluted with EGTA. Flag peptide, or EGTA eluted aliquots (100 µl) were submitted to a combined multidimensional chromatographic-mass spectrometric (MudPIT) analysis as previously described (Graumann et al., 2004; Mayor et al., 2007; Mayor et al., 2005). MudPIT was performed on an LTQ (Figures1D, S1D and Table S2) or LCQ (Figure S1C) mass spectrometer. For MudPIT performed on the LTQ, a Surveyor HPLC (ThermoFisher, Waltham, MA) was coupled online and the separation of peptides was achieved by a ten-step sequence. For MudPIT performed on the LCQ Deca XP, an Agilent 1100 HPLC (Agilent, Santa Clara, CA) was coupled on-line to the LCQ Deca XP. Separation of peptides was achieved with a 6-step method. Data collected by Xcalibur was searched by SEQUEST against the *Saccharomyces* Genome Database (SGD; www.yeastgenome.org), common contaminants were filtered out, e.g. keratins, and DTASelect was used to filter the final results (two-peptide filter).
To isolate 26S proteasomes under denaturing conditions after in vivo crosslinking (Figure 5B), the method developed by Guerrero et al (Guerrero et al., 2008) was followed essentially as described except that cells were lysed in 6M guanidine-HCl. Following binding to Ni-NTA Superflow beads, all subsequent steps were conducted in buffers containing 8 M urea. Eluates from the Ni-NTA beads were bound to Streptavidin magnetic beads (Pierce), washed as described (Guerrero et al., 2008), and bound proteins eluted by boiling in 5X SDS buffer for 15 minutes.

Native Gel Electrophoresis.

Purified 26S proteasomes were resolved by nondenaturing PAGE as described (Verma et al., 2000). Briefly, 4 % polyacrylamide gels were run in the cold room, after which the gel was incubated with the fluorescent peptidase substrate (Succ-LLVY-AMC) at 30°C for 20 minutes. Bands were visualized upon exposure to a UV transilluminator.

Immunoprecipitation analyses to determine interacting proteins.

Overnight cultures were diluted to O.D. 0.2 and grown to an O.D. of 1.0 before being subjected to treatment (such as UV). Cultures were harvested post-treatment, washed, and cell pellets drop-frozen in liquid nitrogen after washing. Cells were thawed in Lysis Buffer (composition described below) at a ratio of 1 ml per100 O.D. unit cells. Glass beads (1 ml) were additionally added, and cells were disrupted using FastPrep-24 at a setting of 6.5 for 90 sec, followed by 5 min cooling on ice, and another cycle of vortexing for 90 sec. Lysates were removed, beads washed with lysis buffer, and pooled lysates were clarified by centrifugation at 14000 rpm (Refrigerated Eppendorf, Model # 5417R) for 15 minutes. Clarified supernatants were bound to anti-epitope beads for 2-3 hours with rotation at 4°C, after which the beads were washed 4 times with buffer containing detergent, and twice with buffer minus detergent. Double-strength SDS buffer was added in equal volume to bead volume and the beads were boiled for 3-5 mins. Boiled aliquots were resolved by SDS-PAGE, transferred to nitrocellulose, immunoblotted with the desired antibody, and developed using either ECL, SuperSignal West Pico, or SuperSignal West Femto (Thermo). Buffer compositions were as below:

26S proteasome Lysis/Wash Buffer B: 50 mM Tris, pH 7.5, 150 mM NaCl, 15% glycerol, 0.2 % Triton, 25 mM b-glycerophosphate, 25 mM NEM, 1X Protease Inhibitor tablet (minus EDTA), 0.5 mM AEBSF, 2 mM ATP, 5 mM $MgCl_2$. This buffer was used for native immunoprecipitations of epitope-tagged proteasome subunits.

For all other non-proteasomal immunoprecipitations, including retrieval of Ub conjugates from extracts on GstDsk2 immobilized on glutathione resin the following buffers were used*:*

Lysis Buffer C: 50 mM HEPES, pH 7.6, 1.0% Triton, 0.1 % deoxycholate, 0.5M potassium acetate, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 25 mM NEM, 1X Protease Inhibitor cocktail (+EDTA; Roche), 0.5 mM AEBSF, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate.

Wash Buffer D: 25 mM Hepes, pH 7.5, 100 mM potassium acetate, 150 mM KCl, 0.25% Tween 20, 10 % glycerol, 50 mM β-glycerphosphate.

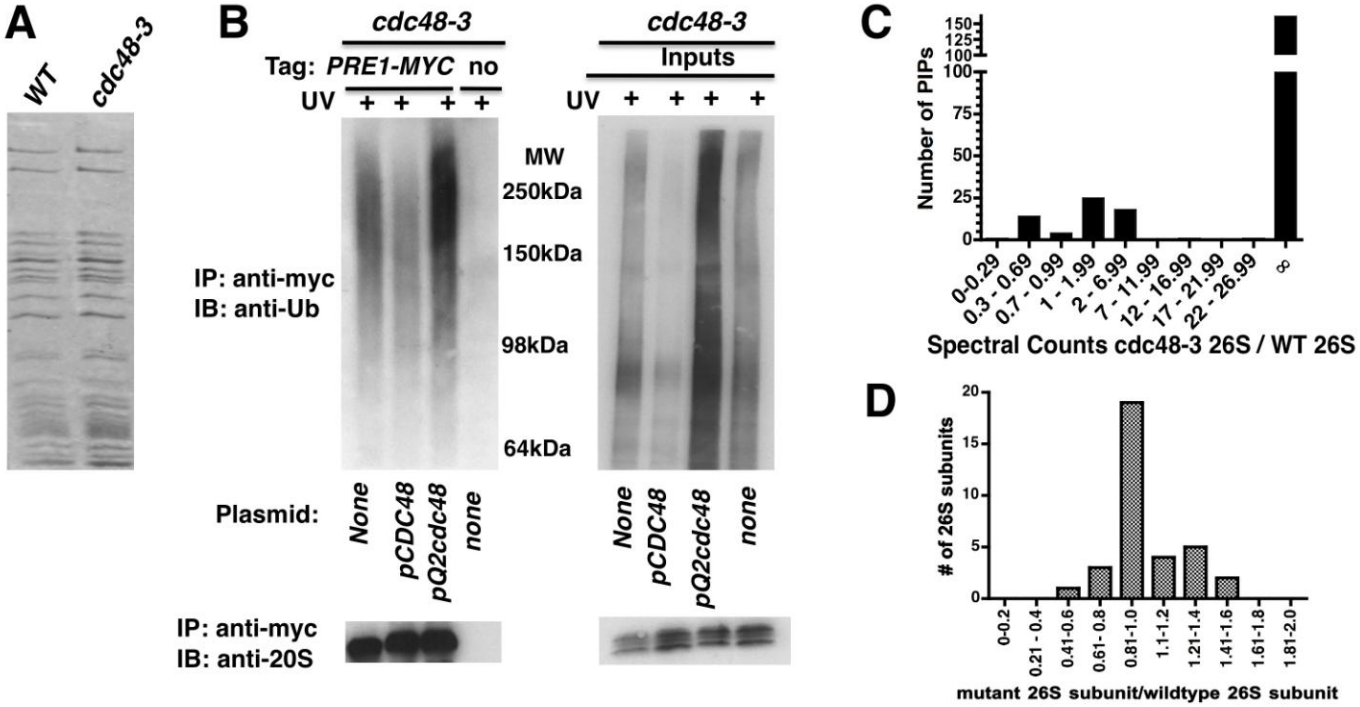

Figure S1, related to Figure 1. Characterization of 26S proteasomes isolated from wildtype (*WT*) and *cdc48* mutant cells (A) No assembly and compositional defect in 26S proteasomes purified from *cdc48-3*. Aliquots of purified preparations were resolved by SDS-PAGE and visualized by staining with Coomassie Blue. (B) ATPase activity of Cdc48 assists in processing of Ub conjugates at 26S proteasomes. Lysates from mutant *PRE1-MYC cdc48-3* cells expressing WT or ATPase-deficient Cdc48 from a plasmid were immunoprecipitated (IP) using anti-myc, and blotted (IB) for Ub and 20S proteasome. (C) MudPIT analysis (on an LCQ mass spectrometer) of 26S proteasome isolated from WT and *cdc48-3* cells. Shown are spectral count ratios for all PIPs. (D) MudPIT analysis of 26S proteasomes isolated from wildtype and *cdc48-3* mutant cells on an LTQ mass spectrometer. Shown are spectral count ratios for 26S subunits.

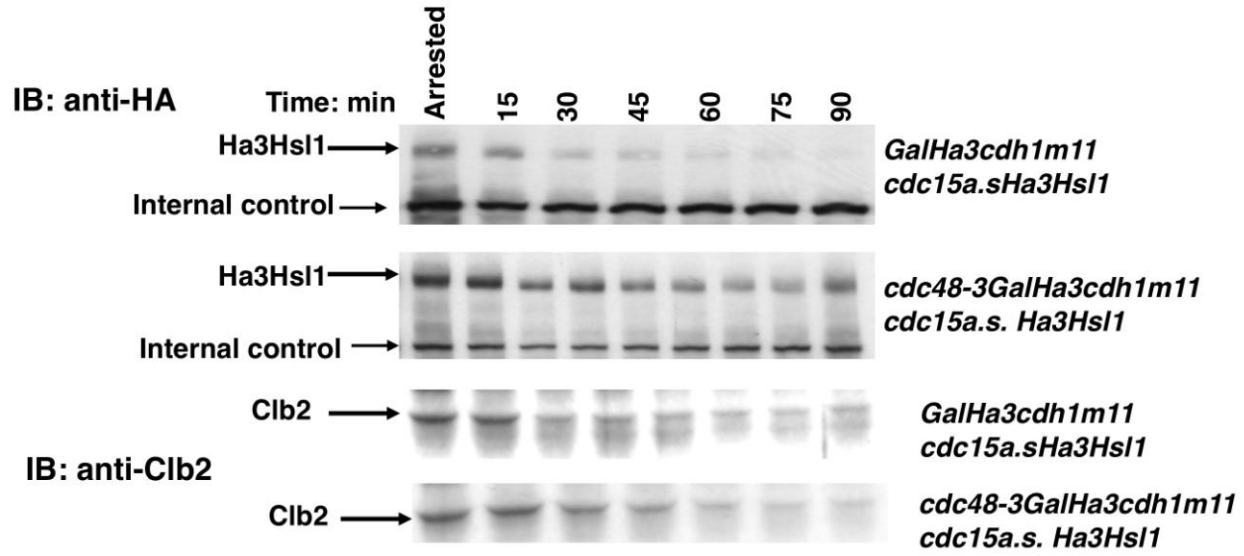

Figure S2, related To Results. Cell cycle-dependent turnover of Hsl1 in G1 is dependent on Cdc48. Analog-sensitive (a.s.) *cdc15* and *a.s.cdc15cdc48-3* cells containing endogenously Ha-tagged Hsl1 and *GALcdh1m11* were grown in YPRaff and arrested with 10 uM ATP analog (1NM-PP1 analogue 9) for 120 min at 25°C. Cells were released into prewarmed YPGal media containing 5 ug/ml alpha-factor at 33°C. Aliquots withdrawn at specified time intervals were processed and immunoblotted (IB) with anti-Ha (Hsl1) and anti-Clb2.

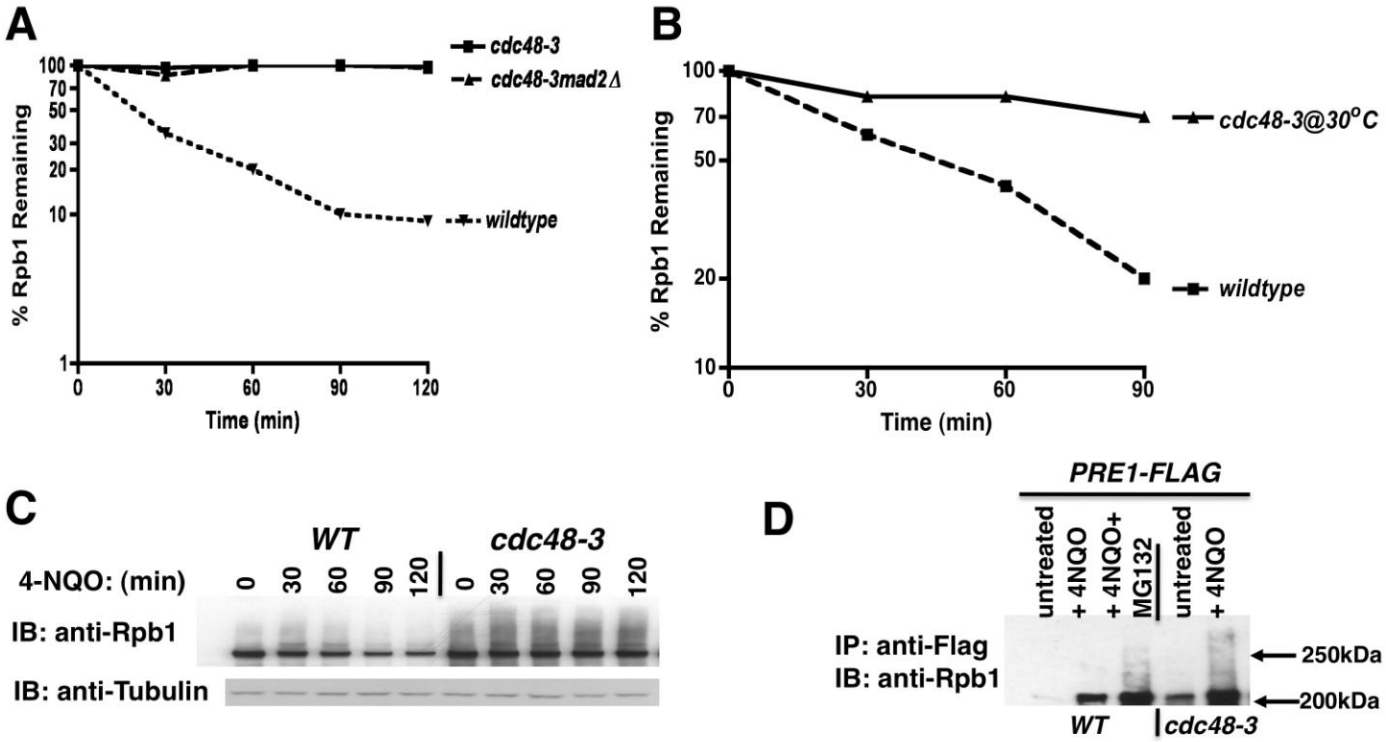

Figure S3, related to Figure 2. Stabilization of Rpb1 in *cdc48-3* mutant cells (A) Wildtype, single, and double mutant cells were arrested at 37°C for one hour before being UV irradiated. Recovery was at 37°C. Quantification on LI-COR was performed following normalization with tubulin (B) As in (A), except overnight cultures grown at 25°C were diluted and grown at 30°C for five hours before being UV irradiated. Recovery was at 30°C. (C) WT and *cdc48-3* cells were shifted to 37°C for 1 hour and then treated with 6 µg/ml 4-NQO and 100 ug / ml cycloheximide for the indicated times (D) Cells were held at 37°C for one hour prior to addition of 6 µg/ml 4-NQO and 40 µM MG132 for another hour, at which point they were harvested and 26S proteasomes isolated.

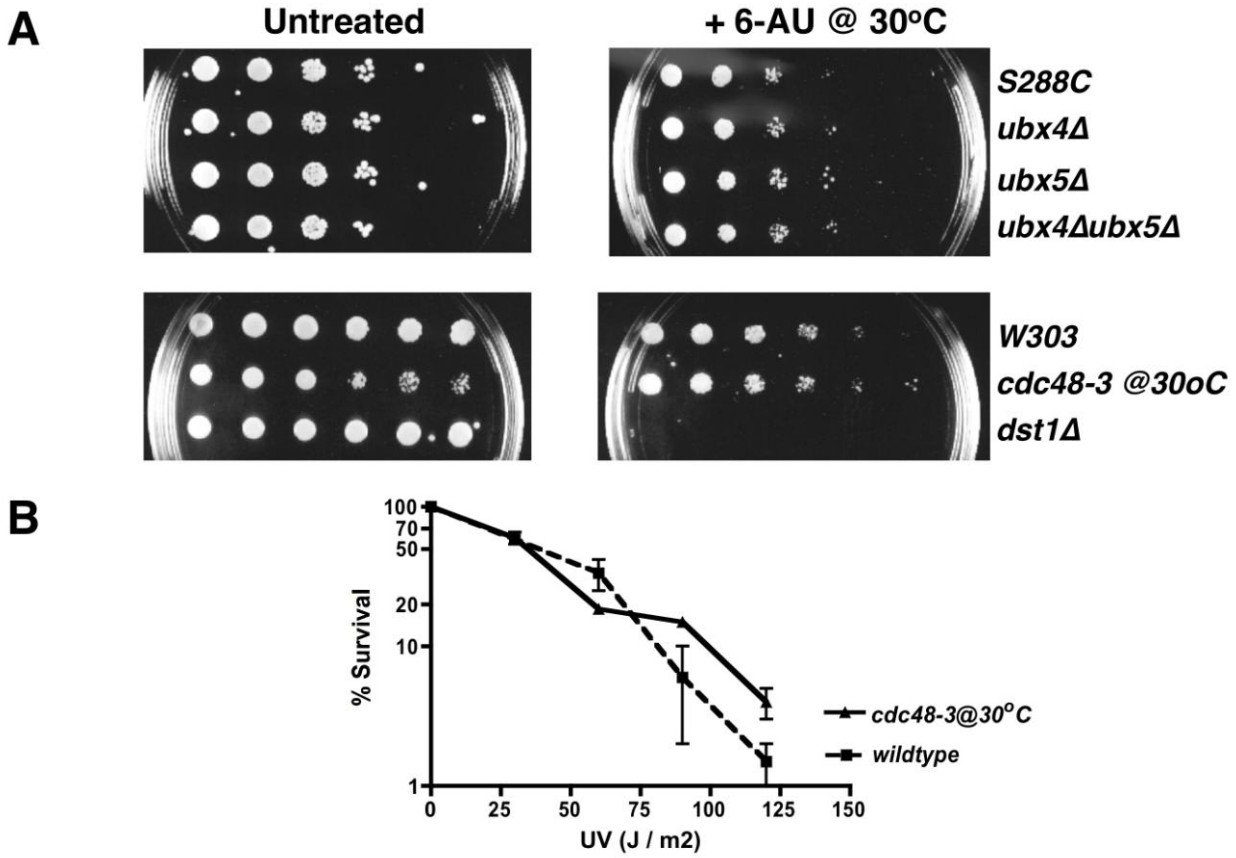

Figure S4, related to Results. (A) Ubx proteins do not play a general role in transcription. Cultures of indicated strains transformed with pRS316 (*URA3*) plasmids were serially diluted on plates containing 100 µg/ml 6-azauracil (AU) and scored after 3 days at 30°C**.** (B**)** Wildtype and *cdc48-3* cells were UV irradiated at the indicated dosages and colonies scored after 3 days of incubation at 30°C in the dark.

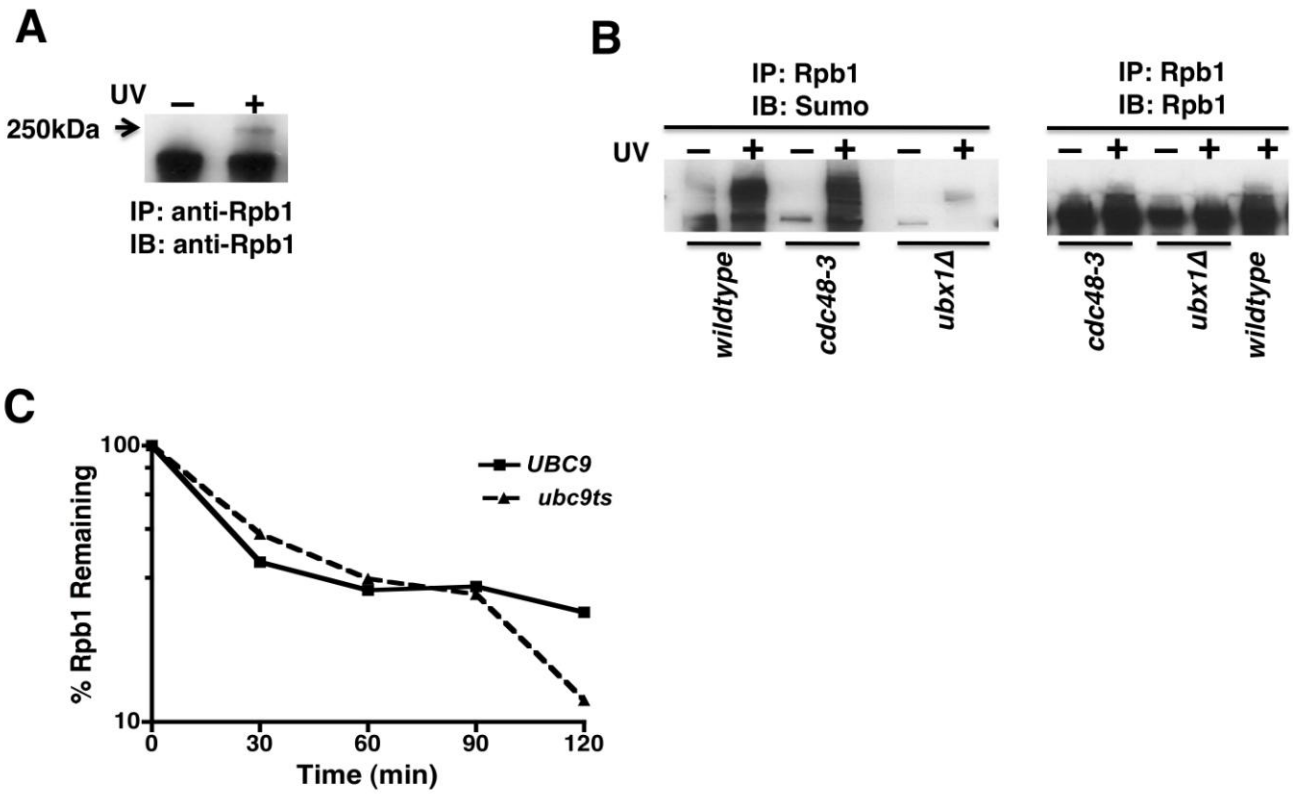

Figure S5, related to Figure 4. Rpb1 is sumoylated after DNA damage, and sumoylation is not required for UV-dependent turnover. Lysates from wildtype cells (A**)** and wildtype and mutant cells (B), either UV-treated or not, were immunoprecipitated (IP) and blotted (IB) with indicated antibodies. (C) UV-dependent turnover of Rpb1 in *ubc9* mutant deficient for sumo-conjugating activity

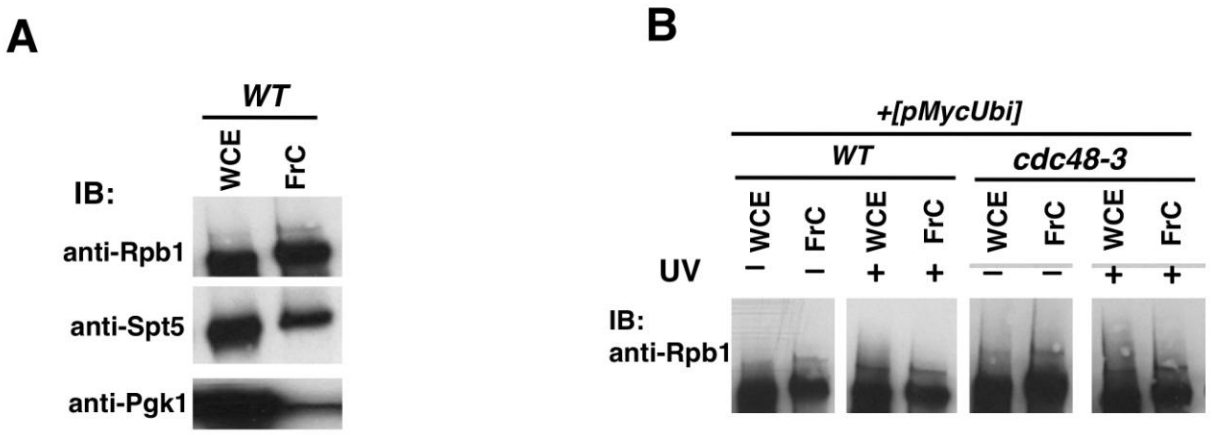

Figure S6, related to Figure 6. (A) Rpb1 fractionated with chromatin in untreated cells, which was de-enriched for cytosolic marker Pgk1. Whole cell extract (WCE) from wildtype (WT) cells was centrifuged through a sucrose cushion to yield a crude pellet that was washed once to yield crude chromatin pellet (FrC). FrC was immunoblotted (IB) for Rpb1 (4H8), transcription factor Spt5, and Pgk1.
(B) Loading Controls for Figure 6C. WT and mutant cells expressing Myc-tagged Ub plasmid were shifted from 25°C to 37°C, and Cu2+ added to induce expression of Ub. Cultures were irradiated with UV (or not), and harvested. Lysates were prepared and centrifuged through a sucrose cushion to yield the Low-speed pellet which was washed once to yield crude chromatin pellet (FrC). FrC was extracted with Benzonase for the experiment in Figure 6C as described in the legend.

Table S1, related to Experimental Procedures. Yeast Strains

Strain	Genotype	Source
RJD360	*can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1, MATa*	This Lab
RJD487	*leu2, ura3, trp1, GAL, MATα*	This Lab
RJD779	*ura3, leu2, trp1, pep4::TRP1, cdc34-2, MATa*	This Lab
RJD1648	*ura3-52, his3-Δ200, lys2-801, ubc9::TRP1, leu2::ubc9Pro-Ser::LEU2*	E. Johnson
RJD1650	*trp1-1, ura3-52, his3-Δ200, leu2-3,-112, lys2-801*	E. Johnson
RJD1721	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, MATa*	Open Biosystems
RJD1869	*can1-100, leu2-3,-112, his3-11,-15, trp1-1, ade2-1, PRE1::PRE1-Flag-His6[URA3], MATa*	This Study
RJD2547	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ufd2::KANMX, MATa*	Open Biosystems
RJD2551	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx1::KANMX, MATa*	Open Biosystems
RJD2589	*ura3-52, leu2Δ1, trp1Δ63, npl4-1, MATa*	P. Silver
RJD2694	*cdc48-3, ura3-52, leu2-3, MATα*	Y. Ye
RJD2719	*can1-100, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade2-1, CDC48::CDC48-HPM[HIS], MATa*	This Study
RJD2775	*his3Δ1, leu2Δ0, met15Δ0,ura3Δ0, NPL4::NPL4-TAP[HIS], MATa*	Open Biosystems
RJD2776	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, UBX1::UBX1-TAP[HIS], MATa*	Open Biosystems
RJD2876	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, UFD1::UFD1-TAP[HIS], MATa*	Open Biosystems
RJD2886	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, CDC48::CDC48-TAP[HIS], MATa*	Open Biosystems
RJD2902	*cdc48-3, ura3-52, leu2-3, PRE1::PRE1-FLAG-His6-URA3, MATα*	This Study
RJD3024	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, UFD3::UFD3-TAP[HIS], MATa*	Open Biosystems
RJD3124	*can1-100, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade2-1, pep4::TRP1, UFD2::UFD2-FLAG[HIS], MATa*	This Study
RJD3166	*his3Δ0, leu2Δ0, met15Δ0, ura3Δ0, ubx2::ubx2-TAP[HIS], MATa*	Open Biosystems
RJD3176	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx2::KANMX, MATa*	Open Biosystems
RJD3177	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx5::KANMX, MATa*	Open Biosystems
RJD3178	*his3Δ0, leu2Δ0, met15Δ0, ura3Δ0, UBX5::UBX5-TAP[HIS], MATa*	Open Biosystems
RJD3264	*his3-Δ200, leu2-3,-112, ura3-52, lys2-801, trp1-1, gal2, ufd1-2, MATa*	M. Hochstrasser
RJD3411	*cdc48-3, leu2-3, his3-11,-15, trp1-1, ura3-1, MATa*	This Study
RJD3437	*pdr5::KANMX, PRE1::PRE1-TEV2-myc9[HIS], bar1::hisG, pep4::TRP1, ura3-1, ade2-1, MATa*	This Study
RJD3454	*cdc48-3, pdr5::KANMX, pre1::PRE1-TEV2-myc9[HIS], bar1::LEU2, pep4::TRP1, ura3-1, ade2-1, MATa*	This Study
RJD4090	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, pdr5::KANMX, MATa*	Open Biosystems
RJD4185	*cdc15 L99G, can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1, hsl1::HSL1-HA[LEU], ura3::GAL-HA3-cdh1-m11URA3, MATa*	This Study
RJD4186	*cdc48-3, cdc15 L99G, can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1, hsl1::HSL1-HA[LEU], ura3::GAL-HA3-cdh1-m11URA3, MATa*	This Study
RJD4214	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, UBX5::UBX5-MYC13[HIS], MATa*	This Study
RJD4436	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, cul3::KANMX, MATa*	Open Biosystems
RJD4472	*rsp5-1, his4-912ΔR5, lys2-128Δ, ura3-52, GAL2+ , MATa*	J. Huibregtse
RJD4496	*can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1, [pRS316], MATa*	This Study
RJD4497	*can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1,cdc48-3, [pRS316-URA3], MATa*	This Study
RJD4503	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx5::KANMX, [pRS316], MATa*	This Study
RJD4523	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, rad26::KANMX, MATa*	Open Biosystems
RJD4570	*his3, leu2, ura3, rad26::KANMX, cdc48-3, MATa*	This Study

RJD4614	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, MATa*	Open Biosystems
RJD4642	*his-, leu2Δ0, ura3, cul3::KANMX, rsp5-1, MATa*	This Study
RJD4669	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx5::KANMX, cul3::KANMX, MATa*	This Study
RJD4680	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, cul3::CUL3-TAP, MATa*	Open Biosystems
RJD4691	*his3, ura3, rsp5-1, ubx5::KANMX, MATa*	This Study
RJD4696	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx5::KANMX, PRE1-Flag-His6::YIPlac211[URA3], MATa*	This Study
RJD4741	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, RPT5::RPT5-HBH-hphmx, MATa*	This Study
RJD4742	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx5::KANMX, RPT5::RPT5-HBH-hphmx, MATa*	This Study
RJD4804	*can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1, [pCUP-MYC-Ubi], MATa*	This Study
RJD4805	*cdc48-3, can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1, [pCUP-MYC-Ubi], MATa*	This Study
RJD4826	*his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, dst1::LEU2, MATa*	F. Winston
RJD4856	*his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, dst1::LEU2, [pRS316] MATa*	This Study
RJD4878	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, pdr5::KANMX, [pGAL-CUL3-His6-HA-ProteinA], MATa*	This Study
RJD4879	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, pdr5::KANMX, UBX5::UBX5-MYC13-HISMX, [pGAL-CUL3-His6-HA-ProteinA], MATa*	This Study
RJD4917	*can1-100, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade2-1, pep4::TRP1, pre1::PRE1-TEV2-myc9[HIS], [pGAL-GST-UBX5-URA3], MATa*	This Study
RJD4966	*cdc48-3, leu2-3, his3-11,-15, trp1-1, ura3-1, [pGAL-His6-CDC48-V5-His6-URA3], MATa*	This Study
RJD4967	*cdc48-3, leu2-3, his3-11,-15, trp1-1, ura3-1, [pGAL-His6-cdc48Q2-V5-His6-URA3], MATa*	This Study
RJD4996	*cdc48-3, PRE1::PRE1-MYC9-TEV2-HIS, [pGAL-His6-CDC48-V5-His6-URA3], leu2-3, ura3, MATa*	This Study
RJD4997	*cdc48-3, PRE1::PRE1-MYC9-TEV2-HIS, [pGAL-His6-cdc48Q2-V5-His6-URA3], leu2-3, ura3, MATa*	This Study
RJD5096	*pdr5::KANMX, bar1::hisG, pep4::TRP1, ura3-1, ade2-1, [pGAL-GST-UBX5-URA3], MATa*	This Study
RJD5246	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx4::KANMX, MATa*	Open Biosystems
RJD5247	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx6::KANMX, MATa*	Open Biosystems
RJD5248	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx7::KANMX, MATa*	Open Biosystems
RJD5249	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx3::KANMX, MATa*	Open Biosystems
RJD5256	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx4::KANMX, PRE1-Flag-His6::YIPlac211[URA3], MATa*	This Study
RJD5259	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx4::KANMX, ubx5::KANMX, MATa*	Open Biosystems
RJD5325	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx4::KANMX, ubx5::KANMX, [pRS316], MATa*	This Study
RJD5326	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx4::KANMX, [pRS316], MATa*	This Study

Plasmids

RDB628	*CUP-MYC-Ubiquitin*	A. Varshavsky
RDB1672	*GST-DSK2 in pGEX-KG*	M. Funakoshi
RDB1842	*HA3-HSL1 in YIP5*	T. Miyakawa
RDB1939	*GAL-His6-CDC48-V5-His6 in pYCNT*	Y. Ye
RDB1941	*GAL-His6- cdc48Q2-V5-His6 in pYCNT*	Y. Ye
RDB2033	*pRS306-HA3-HCT1-m11*	W. Seufert
RDB2041	*pRS306-cdc15-L99G*	D. G. Drubin
RDB2200	*GAL-CYC1-GST-HIS6-UBX5-URA3 in pEGH*	Open Biosytems
RDB2312	*GAL-CUL3-His6-HA-ProteinA in BG1805*	Open Biosytems

BAIT / PREY	CDC 48 HPM	CDC48 TAP	NPL4 TAP	UFD1 TAP	SHP1/ UBX1 TAP	SEL1/ UBX2 TAP	UBX5 TAP	UFD2 FLAG	UFD3 TAP
CDC48	**67**	**66**	**56**	**75**	**57**	**43**	**33**	**64**	**39**
NPL4	**27**	**55**	**48**	**57**		**15**	**5**	**39**	
UFD1	**33**	**43**	**46**	**45**			**3**	**36**	
SHP1/UBX1	**38**	**43**			**34**			**42**	**14**
SEL1/UBX2	**8**	**8**	**8**	**14**		**27**		**18**	
UBX5		**7**	**8**	**8**			**38**		
UBX4		**4**							
UBX6	**11**								
UBX7	**4**	**6**							
UFD3									**59**
UFD2								**52**	

Table S2, related to Results. Mass spectrometric analyses of Cdc48 and its adaptors.
Chromosomally-tagged Cdc48 and adaptors were immunoprecipitated by virtue of their respective epitope tags and analyzed by mass spectrometry. Numbers in the columns represent percent sequence coverage

Supplemental References

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