Cdc48 Chaperone and Adaptor Ubx4 Distribute the Proteasome in the Nucleus for Anaphase Proteolysis*

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Background: Anaphase progression requires efficient degradation of mitotic regulators.
Results: Mutations in the chaperone Cdc48-Ubx4 perturb anaphase proteolysis and proteasome distribution.
Conclusion: Cdc48-Ubx4 maintains an optimal level of proteasomes for anaphase proteolysis.
Significance: The molecular chaperone Cdc48-Ubx4 links proteasome mobilization with mitosis.

The cell cycle transition is driven by abrupt degradation of key regulators. While ubiquitylation of these proteins has been extensively studied, the requirement for the proteolytic step is less understood. By analyzing the cell cycle function of Cdc48 in the budding yeast Saccharomyces cerevisiae, we found that double mutations in Cdc48 and its adaptor Ubx4 cause mitotic arrest with sustained Clb2 and Cdc20 proteins that are normally degraded in anaphase. The phenotype is neither caused by spindle checkpoint activation nor a defect in the assembly or the activity of the ubiquitylation machinery and the proteasome. Interestingly, the 26S proteasome is mislocalized into foci, which are colocalized with nuclear envelope anchor Sts1 in cdc48-3 ubx4 cells. Moreover, genetic analysis reveals that ubx4 deletion mutant dies in the absence of Rpn4, a transcriptional activator for proteasome subunits, and the proteasome chaperone Ump1, indicating that an optimal level of the proteasome is required for survival. Overexpression of Rpn4 indeed can rescue cell growth and anaphase proteolysis in cdc48-3 ubx4 cells. Biochemical analysis further shows that Ubx4 interacts with the proteasome. Our data propose that Cdc48-Ubx4 acts on the proteasome and uses the chaperone activity to promote its nuclear distribution, thereby optimizing the proteasome level for efficient degradation of mitotic regulators.

Budding yeast Cdc48 and its metazoan homolog VCP/p97 are highly conserved, abundant, and essential AAA-ATPases that participate in many cellular functions. The protein contains two characteristic AAA domains termed D1 and D2 that bind and hydrolyze ATP. Energy from ATP hydrolysis alters the conformation of Cdc48/VCP/p97 hexamer, which exerts changes in the structure or assembly of its associated target proteins (1–3). Through specific adaptors, the chaperone activity of Cdc48/VCP/p97 is targeted to components in the ubiquitin-proteasome system, cell cycle control, transcriptional and metabolic regulation, DNA damage response, chromatin remodeling, selective autophagy, and cell death (4).

The adaptors of Cdc48/VCP/p97 include Npl4-Ufd1 complex and a family of proteins containing the ubiquitin regulatory X (UBX)2 domain (5, 6). The UBX domain mediates the interaction with Cdc48/VCP/p97 and is structurally similar to ubiquitin (7). Budding yeast genome predicts seven Ubx members that participate in ubiquitin-dependent degradation of several model substrates (10, 11), their physiological targets are largely elusive.

Cdc48/VCP/p97 is best known to function in ERAD by extracting misfolded proteins from endoplasmic reticulum (ER) for subsequent degradation by the proteasome (12). This function is mediated by Npl4-Ufd1 adaptor complex that contains a ubiquitin-binding domain for the recognition of ubiquitylated substrates (13, 14). Thus, Cdc48/VCP/p97 is regarded as a ubiquitin-selective chaperone that segregates ubiquitylated proteins from non-modified partners (15). Cdc48/VCP/p97 also exerts its segregase activity on a variety of targets in other cellular processes.

Besides segregating specific ubiquitylated proteins for degradation, Cdc48/p97/VCP appears to broadly affect the ubiquitin-proteasome system. As a substoichiometric component of the proteasome, Cdc48/p97/VCP facilitates the transfer of ubiquitylated substrates to the proteasome (16). Mature 26S proteasome is composed of catalytic 20S core particle (CP) capped by 19S regulatory particle (RP). In budding yeast, the proteasome is largely localized in the nucleus (17). CP and RP are formed as precursor complexes in the cytoplasm and imported into the nucleus independently of each other, and final maturation of the 26S proteasome occurs in the nucleus (18, 19). The assembly of complete proteasome from subcom-

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2 The abbreviations used are: UBX, ubiquitin regulatory X; ERAD, endoplasmic reticulum- associated protein degradation; CP, core particle, RP, regulatory particle; APC/C, anaphase promoting complex/cyclosome; TAP, tandem affinity purification; FRAP, fluorescence recovery after photobleaching.
plexes is an orderly event orchestrated by specific chaperones (20).

Despite its function in proteostasis, Cdc48 was initially isolated as a metaphase mutant in the budding yeast (21). The metaphase-to-anaphase transition normally requires the E3 ubiquitin protein ligase anaphase-promoting complex/cyclosome (APC/C) for the degradation of Securin (Pds1 in budding yeast), mitotic cyclin (Cln2 in budding yeast), and APC/C activator Cdc20. The APC/C activity is blocked by the spindle checkpoint mechanism until metaphase is achieved. Cdc48 is important for metaphase by promoting nuclear localization of type I protein phosphatase (8). In addition, Cdc48 is required for anaphase onset by stabilizing Separase in fission yeast (22), and VCP/p97 antagonizes Aurora B for proper chromosome congression and segregation in animal cells (23).

In this report, we study the function of budding yeast Ubx4 and reveal that Cdc48-Ubx4 is important for mitotic progression. Mutations in Cdc48 and Ubx4 do not directly affect the cell cycle machinery. Rather, they perturb proteasome distribution that impacts on the degradation of mitotic regulators.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids and Yeast Strains**—All strains are derivatives of W303. Gene deletions, epitope tagging, and \( P_{GAL}^{+} - 3HA - UBX4 \) were generated by PCR-mediated integration (24). \( PDS1 - 8myc \) and \( mad2A \) were generated as described (8). For 3Flag-tagged wild-type and truncated Ubx4, the promoter region (500 bp upstream of start codon) of \( UBX4 \) was cloned at HindIII and BamHI in pRS306, the coding region of full-length, N-terminal (ubx4\(^{58} \), amino acids 1–225) or C-terminal region (ubx4\(^{226} \), amino acids 226–416) of \( UBX4 \) at BamHI and XhoI, 3Flag tag at XhoI and Sall, and 500 bp downstream of stop codon at Sall and NotI. The plasmids were linearized with StuI and integrated at the RPS loci.

**Growth of Yeast**—Cells were grown in synthetic medium containing 1% yeast extract, 2% bacto-peptone, and 2% glucose (YPD) or galactose (YPGal). For microscopy studies, cells were grown in synthetic medium containing 0.67% yeast nitrogen base (YNB), complete supplement mixture (CSM, Bio101, Vista, CA) of amino acids or CSM without specific amino acid for selection, and 2% glucose or galactose. For the synchronized cell cycle, cells were grown in YPGal to mid-log phase at 25 °C and then incubated with 1 \( \mu \)g/ml of \( \alpha \)-factor (Sigma) for 3 h to arrest cells at G1 before shifting to YPD plus \( \alpha \)-factor for another 3 h. To release from G1 arrest, cells were washed three times with H\(_2\)O and then resuspended in YPD. \( \alpha \)-factor was added back after >90% of cells had budded to block the second cell cycle. For cycloheximide chase experiments, cycloheximide (100 mg/ml stock in DMSO) was added to 1 mg/ml.

**Yeast Cell Lysates and Western Blots**—Cell lysate preparation for SDS-PAGE and Western blots were performed as described (8). The following antibodies were used: anti-Myc (9E10; Covance, Princeton, NJ), anti-HA (16B12; Covance), anti-Cdc20 (sc-6731; Santa Cruz, Dallas, TX), anti-ycbl2 (sc-9071; Santa Cruz), anti-Flag (M2; Sigma), and anti-Actin (MAB1501; Chemicon, Billerica, MA). Anti-mCherry and anti-GFP antibodies were from Chao-Wen Wang laboratory (IPMB, Academia Sinica).

**Proteasome Purification**—One liter of cells expressing Pre6-TAP were resuspended in native gel lysis buffer (20 mm Tris-HCl, pH 7.4, 5 mm MgCl\(_2\), 1 mm ATP, and 1 mm DTT) and lysed by grinding in liquid nitrogen. Equal amounts of lysates were incubated with IgG Sepharose (GE Healthcare, Pittsburgh, PA) at 4 °C for 2 h. The beads were then washed four times with native gel lysis buffer and once with TEV buffer (50 mm Tris-HCl, pH 8.0, 0.5 mm EDTA, 1 mm DTT), followed by incubation with 100 units of TEV protease (Invitrogen, Carlsbad, CA) overnight at 4 °C. The TEV-released fraction was supplemented with 5 mm CaCl\(_2\) and incubated with calmodulin-Sepharose 4B (Amersham Biosciences, Pittsburgh, PA) at 4 °C for 2 h. The beads were then washed 4 times with Calmodulin wash buffer (10 mm Tris-HCl, pH 8.0, 150 mm NaCl, 10% glycerol, 3 mm DTT, 1 mm MgCl\(_2\), 2 mm CaCl\(_2\), 0.1% Nonidet P-40) and another four times with 25 mm NH\(_4\)HCO\(_3\), followed by denaturation in SDS-PAGE sample buffer. 10% of the sample was loaded onto a 10–20% gradient gel and stained by Sypro Ruby (Invitrogen, Carlsbad, CA). The gel was scanned with Typhoon FLA 9000 (GE Healthcare, Pittsburgh, PA).

**Proteasome Non-denaturing Gel**—Cells at mid-log phase were collected and lysed by bead-beating 5 times for 1 min each with 3 min interval in lysis buffer containing 50 mm Tris, pH 8.0, 5 mm MgCl\(_2\), 1 mm DTT, 1 mm ATP, 10% glycerol, 1 mm PMSF, 10 \( \mu \)g/ml each of leupeptin, pepstatin, and chymostatin. The samples were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C. The protein concentration in the supernatant was measured and adjusted to 10 \( \mu \)g/ml. A 5× loading dye (250 mm Tris, pH 8.0, 50% glycerol, 0.02% bromphenol blue) was added to the lysate, and 2 \( \mu \)l of the samples were loaded onto 4% acrylamide gel made in the non-denaturing gel buffer (50 mm Tris, 50 mm boric acid, 5 mm MgCl\(_2\), 1 mm ATP, and 1 mm DTT). The gel was run in the non-denaturing gel buffer at 100 V for 4.5 h at 4 °C, and then transferred to nitrocellulose membrane for Western blotting with anti-Cp (BML-PW9355; Enzo Life Sciences, Farmingdale, NY), anti-Rpn5 (ab79773; Abcam, Cambridge, UK), and anti-Blm10 (BMW-PW0570; Enzo Life Sciences, Farmingdale, NY).

**In-gel Proteasome Activity Assay**—Yeast spheroplasts were prepared and lysed by bead-beating in native gel lysis buffer. Lysate of 100 \( \mu \)g of total protein was resolved by 4% non-denaturing gel. The gel was run at 100 V for 4 h at 4 °C and then incubated in 5 ml of 50 mm Suc-LLVY-AMC in buffer A (25 mm Tris-HCl, pH 7.4, 10 mm MgCl\(_2\), 1 mm ATP, 1 mm DTT, and 10% glycerol) at 30 °C for 30 min. Immediately after visualization of the fluoresce signals of RP-CP and RP-CP-RP by UV transilluminator (UVP 600, Upland, CA), SDS was added to 0.02% in buffer A to detect CP.
Fluorescence Microscopy—Images were taken using a 100 × 1.4 N/A lens and CoolSNAP HQ2 CCD camera (Photometrics, Tucson, AZ) on an Olympus IX71 fluorescence microscope controlled by DeltaVision system (Applied Precision, Issaquah, WA). Z-stacks of 13 optical sections with spacing of 0.4 μm were collected and processed by Softworx software.

FRAP Analysis—The experiment was carried out on Zeiss LSM510 META confocal microscope equipped with EC Plan-NEOFLUAR 40×/1.3 objective lens and an objective heater to maintain the sample at 25 °C. A 0.9 milliwatt argon laser was set to an extreme power (100% of laser power) to measure the fluorescence intensity before photobleaching, followed by 10 iterative laser pulses at full power to photobleach a circular area of ~1.144 μm in diameter in the nucleus. Fluorescence recovery was monitored every 500 ms. The fluorescence intensity values were exported from LSM software (Carl Zeiss, Oberkochen, Germany) to Excel. Intensity (Fs) was corrected for background noise by subtracting the intensity in a nearby region without any cell (FB) and normalized by the spontaneous photobleaching rate (r), which is determined by comparing the fluorescence of the control region before (FC0) and after (FC) photobleaching (r = FC/FC0). The normalized intensity F = (Fs-FB)/r. The recovery t1/2 was calculated by Zeiss FRAP software and expressed as average ± S.D.

Cim5-TAP Pull-down—The pull-down was carried out as described (25). Briefly, yeast cultures at A600 of 1–2 were harvested by centrifugation. Cell pellets were washed in ice-cold water and lysed with native lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 15% glycerol, 25 mM β-glycerophosphate, 25 mM NEM, 10 μg/ml each of leupeptin, pepstatin, and chymostatin, 2 mM ATP, 5 mM MgCl2, and 2 mM CaCl2) by mixing the cell pellet with an equal volume of Zirconia beads and beating for 1 min at 4 °C. Lysates were centrifuged by centrifugation at 15,000 rpm for 5 min at 4 °C. The supernatants were mixed with calmodulin-Sepharose 4B (Amersham Biosciences) for 3 h at 4 °C. The beads were washed four times with native lysis buffer containing 0.2% Triton X-100 and twice with wash buffer containing 25 mM Tris, pH 7.5, 10 mM MgCl2, 2 mM ATP. The bound proteins were then solubilized by heating to 95 °C in SDS-PAGE sample buffer.

In Vitro APC/C Assay—APC/C subunit Cdc16 was tagged with TAP and purified sequentially by IgG-Sepharose and then by calmodulin-Sepharose beads as described previously (26). APC activator Cdc20 and the substrate Pds1 were transfected and translated in vitro with TNT T7-coupled reticulocyte lysate system (Promega). For Pds1, [35S]methionine was added during
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Mitotic Delay in cdc48-3 ubx4 Cells Is Independent of the Spindle Checkpoint and APC/C—Pds1, Cdc20, and Cdc42 are ubiquitylated by APC/C after anaphase onset. We have previously shown that cdc48-3 at non-permissive temperature affects kinetochore bi-orientation, leading to activation of the spindle checkpoint and block of anaphase onset (8). Thus, the delayed degradation of APC/C substrates in cdc48-3 and cdc48-3 ubx4 mutants at permissive temperature may be due to partial activation of the spindle checkpoint. We thus asked whether deletion of the evolutionarily conserved spindle checkpoint gene MAD2 may suppress the degradation defect. We did not include Pds1 in this experiment, because the effect of cdc48-3 ubx4 double mutations on Pds1 was minor compared with Clb2 and Cdc20 (Fig. 1B). This result shows that cdc48-3 ubx4 cells are delayed in mitosis.

We next examined the levels of various mitotic regulators by Western blots to determine what stage of mitosis was affected in cdc48-3 ubx4 cells. The anaphase inhibitor Pds1 that accumulates from S to M phase and is degraded after anaphase onset showed a similar pattern of expression during the synchronized cell cycle in wild-type and ubx4 cells (Fig. 1C). Pds1 also accumulated with similar kinetics in cdc48-3 and cdc48-3 ubx4 mutants, but its decline was slightly delayed in cdc48-3 and further delayed in cdc48-3 ubx4 cells (Fig. 1C). Similarly, mitotic cyclin Clb2 and APC/C activator Cdc20 disappeared with slower kinetics in cdc48-3 and cdc48-3 ubx4 than in wild-type and ubx4 cells (Fig. 1C). These results show that cdc48-3 ubx4 cells are delayed at the metaphase-anaphase transition.

Mitotic Delay in cdc48-3 ubx4 Is Independent of the Spindle Checkpoint and APC/C—Pds1, Cdc20, and Cdc20 are ubiquitylated by APC/C after all kinetochores are properly attached to spindle microtubules. We have previously shown that cdc48-3 at non-permissive temperature affects kinetochore bi-orientation, leading to activation of the spindle checkpoint and block of anaphase onset (8). Thus, the delayed degradation of APC/C substrates in cdc48-3 and cdc48-3 ubx4 mutants at permissive temperature may be due to partial activation of the spindle checkpoint. We thus asked whether deletion of the evolutionarily conserved spindle checkpoint gene MAD2 may suppress the degradation defect. We did not include Pds1 in this experiment, because the effect of cdc48-3 ubx4 double mutations on Pds1 was minor compared with Clb2 and Cdc20 (Fig. 1C). Western blots show that MAD2 deletion did not accelerate the decline of Clb2 and Cdc20 levels after mitosis in cdc48-3 ubx4 cells.

FIGURE 2. Clb2 and Cdc20 degradation defect in cdc48-3 ubx4 double mutant is independent of the spindle checkpoint. A, MAD2 deletion does not rescue the degradation defect in cdc48-3 ubx4. Cells were first arrested at G1 by α-factor and then released into the cell cycle in YPD as described in Fig. 1. Samples were taken at the indicated times for Western blot with anti-Clb2 and anti-Cdc20 antibodies. Western blot of actin serves as loading control. B, cdc48-3 ubx4 double mutants affect Cdc20 degradation at G1. Cells expressing 4myc-Cdc20 were arrested at G1 by α-factor first in YPGal and then in YPD. Samples were taken immediately before and at the indicated times after the addition of cycloheximide (CHX), followed by Western blot with anti-4myc and anti-actin antibodies. The plot shows the average and standard deviation of three independent experiments with protein levels normalized to that in time 0.

the reaction. APC/C assays were performed as described (26). The reactions were incubated at 25 °C, and aliquots were taken and stopped by the addition of SDS-PAGE sample buffer at indicated times. Samples were resolved by SDS-PAGE, and the dried gel was exposed to a Phosphor screen (GE Healthcare, Pittsburgh, PA), which was then scanned by Typhoon FLA 9000 (GE Healthcare) and quantified by ImageQuant software.

RESULTS

cdc48-3 ubx4 Double Mutant Is Delayed in Mitosis—Cdc48 functions in diverse cellular processes through specific adaptors. The UBX domain-containing adaptor Ubx1/Shp1 is required for mitotic progression (8). Unlike UBX1, the other UBX members, UBX2~7, are not essential for vegetative cell growth. Interestingly, we found that deletion of UBX4 was synthetic-lethal with the temperature-sensitive cdc48-3 mutant (data not shown). To determine the terminal phenotype of cdc48-3 ubx4 double mutants, we conditionally expressed PGAL-3HA-UBX4 promoter and added HA3 tag at the N terminus of Ubx4. The resulting strain PGAL-3HA-UBX4 with or without cdc48-3 mutation can grow on galactose-containing plate, whereas cdc48-3 PGAL-3HA-UBX4 strain cannot survive on the glucose-containing plate even at permissive temperature for cdc48-3 (Fig. 1A). This result is consistent with the synthetic lethality of cdc48-3 ubx4Δ. We hereafter refer to PGAL-3HA-UBX4 and cdc48-3 PGAL-3HA-UBX4 cells shifted from galactose to glucose medium as ubx4 and cdc48-3 ubx4, respectively.

To determine whether cdc48-3 ubx4 cells had any defect in cell cycle progression, we measured their DNA content in a synchronized cell cycle after release from G1 arrest by α-factor. The fluorescence-activated cell sorter (FACS) analysis shows that cdc48-3 ubx4 cells maintained 2N DNA content even at 4 h after release into the cell cycle, while the majority of wild-type, cdc48-3, and ubx4 cells had exited mitosis (Fig. 1B). This result shows that cdc48-3 ubx4 cells are delayed in mitosis.

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Mitotic Delay in cdc48-3 ubx4 Is Independent of the Spindle Checkpoint and APC/C—Pds1, Cdc20, and Cdc20 are ubiquitylated by APC/C after all kinetochores are properly attached to spindle microtubules. We have previously shown that cdc48-3 at non-permissive temperature affects kinetochore bi-orientation, leading to activation of the spindle checkpoint and block of anaphase onset (8). Thus, the delayed degradation of APC/C substrates in cdc48-3 and cdc48-3 ubx4 mutants at permissive temperature may be due to partial activation of the spindle checkpoint. We thus asked whether deletion of the evolutionarily conserved spindle checkpoint gene MAD2 may suppress the degradation defect. We did not include Pds1 in this experiment, because the effect of cdc48-3 ubx4 double mutations on Pds1 was minor compared with Clb2 and Cdc20 (Fig. 1C). Western blots show that MAD2 deletion did not accelerate the decline of Clb2 and Cdc20 levels after mitosis in cdc48-3 ubx4 cells.
cells to the kinetics in cdc48-3 (Fig. 2A). Deletion of another spindle checkpoint gene MAD3 also gave the same result (data not shown). Therefore, the anaphase delay of cdc48-3 ubx4 was not due to activation of the spindle checkpoint.

Cdc20 is rapidly turned over from late mitosis until G1 phase. We thus determined if cdc48-3 ubx4 double mutations also affect Cdc20 protein stability at G1. We used cells deleted for MAD2 to avoid any effect from the spindle checkpoint that may interfere with G1 arrest. Because Cdc20 exists at a low level at G1, we expressed 4myc-tagged Cdc20 to enhance detection. After adding cycloheximide to block protein synthesis at G1, 4myc-Cdc20 disappeared by 5 min in mad2Δ and ubx4 mad2Δ, whereas about 40 and 60% of original 4myc-Cdc20 level remained in cdc48-3 mad2Δ and cdc48-3 ubx4 mad2Δ, respectively (Fig. 2B). In addition, the steady-state level of 4myc-Cdc20 (time 0, before cycloheximide addition) was elevated in cdc48-3 ubx4 mad2Δ (Fig. 2B). These results show that cdc48-3 ubx4 double mutations delayed Cdc20 degradation at G1, supporting the notion that the degradation defect of APC/C substrates in cdc48-3 ubx4 mutant is independent of the spindle checkpoint or a failure to exit mitosis.

We next asked whether cdc48-3 ubx4 mutations may perturb APC/C function. We performed tandem affinity purification (TAP) on the APC/C subunit Cdc16 and found that the associated APC/C components were essentially the same between wild-type and cdc48-3 ubx4 cells (Fig. 3A), indicating that the mutations did not affect the assembly of APC/C. Furthermore, in vitro APC/C assay shows that APC purified from wild-type and cdc48-3 ubx4 cells ubiquitylated the substrate [35S]Pds1 to the same degree and with similar kinetics (Fig. 3B), indicating that APC/C activity was intact. Therefore, the inability to efficiently degrade Cdc20 and Cdc20 in cdc48-3 ubx4 is not caused by a defect in APC/C.

cdc48-3 ubx4 Mutations Have No Gross Effect on Proteasome Assembly or Activity—We next determined whether cdc48-3 ubx4 double mutations affect proteasome function. TAP of Pre6, α4 subunit of the CP, shows that proteins associated with Pre6-TAP were similar in cdc48-3 and cdc48-3 ubx4 cells (Fig.
Mass spectrometry analysis further confirmed that the protein complex consisted of subunits in the RP and the CP (data not shown), indicating that cdc48-3 ubx4 mutations do not affect the assembly of 26S proteasome. We also analyzed proteasome architecture by Western blotting of non-denaturing gels. We used anti-Rpn5 antibody to detect the RP and found that the levels of the CP associated with one (RP-CP) or two RP (RP-CP-RP) were similar between wild-type, cdc48-3, ubx4, and cdc48-3 ubx4 cells (Fig. 4B). The Western blot of Blm10, another CP activator (27), also shows comparable levels of RP-CP-Blm10, Blm10-CP-Blm10, and Blm10-CP among these strains (Fig. 4B). Consistent with these results, Western blotting with anti-CP antibody shows that cdc48-3 ubx4 mutations did not affect the assembly of CP with RP and Blm10 (Fig. 4C).
Furthermore, in-gel peptidase assay using the fluorogenic substrate Suc-LLVY-AMC shows comparable activities of RP-CP-RP and RP-CP between wild-type, *cdc48-3*, *ubx4*, and *cdc48-3 ubx4* cells (Fig. 4C). Incubation of the non-denaturing gel with SDS to allow access to the catalytic core of the CP shows similar activities of CP and Blm10-CP among these strains (Fig. 4C). Together, these results suggest that *cdc48-3 ubx4* double mutations do not affect the structural integrity and the activity of 26S proteasome.

To investigate the effect of *cdc48-3 ubx4* double mutations on the ubiquitin-proteasome system *in vivo*, we examined several unstable proteins, including the model substrates Deg1-GFP, Deg-NLS-GFP (Deg1-GFP with nuclear localization signal), CPY*-HA, and G1 regulator Far1. Except for the ERAD
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FIGURE 6. Pre6 is congregated with Sts1 at nuclear periphery in cdc48-3 ubx4 cells. A, Pre6-GFP foci reside at the inner side of the nuclear envelope. cdc48-3 P_{GAL}-3HA-UBX4 cells expressing Pre6-GFP and Nup49-mCherry were grown in galactose-containing medium. Maximum projections of Z-stack images are shown. Bar, 2 μm. B, Sts1-mCherry levels are not affected by cdc48-3 or cdc48-3 ubx4 mutations. Two independent isolates of the indicated strains expressing Sts1-mCherry and Pre6-GFP were analyzed by Western blotting with anti-mCherry and anti-actin antibodies. Actin serves as loading control. C, Cdc48 or Cdc48QQ was expressed from GAL1 promoter in cells expressing Pre6-GFP and Sts1-mCherry. Maximum projections of Z-stack images are shown. Bar, 2 μm.

A possibility exists that the clustering of Sts1 and the proteasome is caused by misregulation of Sts1. Based on the fact that fission yeast Cut8 is a short-lived protein that is degraded upon polyubiquitylation and association with the proteasome (28), we asked whether Sts1 protein level might be altered by cdc48-3 ubx4 mutations. Western blot result shows that Sts1-

...substrate CPY*-HA, none of the other substrates were significantly stabilized in cdc48-3 ubx4 cells (data not shown). Thus, cdc48-3 ubx4 double mutations do not have a general effect on the degradation of short-lived proteins, consistent with the notion that both structure and activity of the proteasome are intact. Nevertheless, tetrad analysis shows that ubx4Δ was synthetic-lethal with deletion of both CP chaperone UMP1 (Fig. 4D) and RPN4, the transcriptional activator for proteasome subunits (Fig. 4E), indicating that the mutant was sensitive to perturbation of proteasome biogenesis.

Proteasomes Form Nuclear Foci in cdc48-3 ubx4—We next examined the subcellular distribution of proteasomes by tagging Pre6 with GFP. Pre6-GFP was largely localized in the whole nucleus of wild-type and ubx4 cells (Fig. 5A). Interestingly, ~50% of cdc48-3 cells in glucose medium showed a small puncta of Pre6-GFP signal at the nuclear periphery in addition to the signal in the nucleoplasm (Fig. 5, A and B). The small Pre6-GFP puncta also existed in cdc48-3 P_{GAL}-3HA-UBX4 cells grown in galactose-containing medium (Fig. 5A). However, upon depletion of Ubx4 by glucose, ~80% of cdc48-3 P_{GAL}-3HA-UBX4 cells showed one dot of Pre6-GFP signal that was larger and brighter than the puncta found in cdc48-3 (Fig. 5, A and B). Similarly, GFP-tagged Pre2, β5 subunit of CP, and RP subunits Cim5 and Rpn11 also formed foci in cdc48-3 ubx4 cells (Fig. 5C). These results suggest that the 26S proteasome was abnormally clustered at nuclear periphery.

We further examined whether Pre6-GFP foci in cdc48-3 ubx4 cells might represent stable aggregates of the proteasome. We performed fluorescence recovery after photobleaching (FRAP) analysis and found that nuclear Pre6-GFP in wild-type cells was quickly recovered upon photobleaching with $t_{1/2}$ ~0.66 s (Fig. 5D), indicating that the proteasome is highly mobile in the nucleus. Photobleaching the Pre6-GFP foci in cdc48-3 ubx4 cells showed a slightly slower recovery rate of $t_{1/2}$ ~1.45 s (Fig. 5D), suggesting that Pre6-GFP foci are dynamic structures, not stable aggregates.

Proteasomes Congregate with Nuclear Envelope Anchor Sts1 in cdc48-3 ubx4—By labeling the nuclear envelope with mCherry-tagged nuclear pore complex subunit Nup49, we found that Pre6-GFP foci localized to the nucleoplasmic side of the nuclear envelope in cdc48-3 ubx4 cells (Fig. 6A). Thus, Cdc48-Ubx4 is important for nuclear distribution, but not import, of the proteasome.

Previous study has shown that the fission yeast nuclear membrane protein Cut8 provides an anchor for the proteasome to enrich at the nuclear periphery (28). Our result of proteasome foci at the nuclear envelope of cdc48-3 ubx4 cells suggests that the proteasome might be retained at the nuclear membrane. We thus examined the localization of Sts1, a budding yeast homolog of Cut8, by tagging the protein with mCherry. Sts1-mCherry signal was weak and mostly localized to the nucleus in both wild-type and ubx4 cells (Fig. 6B), but it appeared as a small dot at the nuclear periphery in a fraction of cdc48-3 cells (Fig. 6B). The Sts1-mCherry dot was co-localized with Pre6-GFP and became larger and brighter in cdc48-3 ubx4 cells (Fig. 6B), showing that Sts1 and the proteasome were congregated together in the mutants.
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A

![Image](https://example.com/image1)

B

![Image](https://example.com/image2)

FIGURE 7. Overexpression of Rpn4 suppresses cdc48-3 ubx4 phenotypes. A, cells carrying high-copy 2μ plasmid pRS425 without (−) or with (+) RPN4 were spotted in 10-fold serial dilutions from left to right on selection plates containing galactose or glucose. B, cells without (−) or with (+) high-copy RPN4 were first arrested at G1 and then released into cell cycle as described in Fig. 1. Samples were taken at the indicated times for Western blot analysis of Clb2 and Cdc20. Actin serves as loading control.

mCherry protein levels were similar in wild-type, cdc48-3, ubx4, and cdc48-3 ubx4 cells (Fig. 6C), indicating that the formation of Sts1 and proteasome foci was not caused by a change of Sts1 protein stability.

We speculated that Cdc48 may employ its chaperone activity to distribute the proteasome in the nucleus. We tested this possibility by overexpressing dominant-negative Cdc48Q52 mutant that blocks ATP hydrolysis (13). Induction of Cdc48Q52, but not wild-type Cdc48, through GAL1 promoter caused a metaphase arrest (data not shown). Upon Cdc48Q52 expression, Pre6-GFP formed perinuclear foci similar to that in cdc48-3 ubx4 cells (Fig. 6D). The foci were also co-localized with Sts1-mCherry signal (Fig. 6D). This result shows that ATP hydrolysis of Cdc48 is important for proteasome distribution in the nucleus.

Rpn4 Overexpression Rescues the Phenotypes in cdc48-3 ubx4—We performed a high-copy suppressor screen and identified RPN4 that on a 2μ plasmid rescued the viability of cdc48-3 Pgal-3HA-UBX4 cells on the glucose-containing plate (Fig. 7A). Consistent with the cell growth, a high copy of RPN4 advanced the degradation of Clb2 and Cdc20 proteins in cdc48-3 ubx4 mad2Δ cells to similar kinetics as in cdc48-3 mad2Δ (Fig. 7B), although it did not restore normal proteasome distribution (data not shown). RPN4 encodes a transcription factor that binds the promoter of many genes in the ubiquitin-proteasome system and up-regulates the expression of proteasome subunits when proteasome function is compromised (29, 30). The result suggests that elevating the proteasome concentration can overcome the defect caused by proteasome clustering, supporting the notion that anaphase proteolysis requires an optimal level of the proteasome.

Ubx4 Associates with the Proteasome—Cdc48 may disassemble the proteasome from its anchor at the nuclear envelope to mobilize the proteasome in the nucleus. It is possible that Ub4 mediates this chaperone function of Cdc48 by interacting with the proteasome. Ub4 contains a putative ubiquitin-like (UBL) domain at its N terminus and a UBX domain at the C terminus (Fig. 8A). UBL domain shares high similarity with ubiquitin and is a proteasome-binding motif in other proteins (31). We first determined which region of Ub4 was required for viability in cdc48-3 strain. We expressed 3Flag-tagged Ub4, Ub4N (amino acids 1–225), or Ub4C (amino acids 226–416) in cdc48-3 Pgal-3HA-UBX4 strains and found that only full-length Ub4-3Flag, but not the truncated proteins, supported cell growth on glucose-containing plate (Fig. 8B). This result shows that both UBL and UBX domains are important for the function of Ub4. To determine if Ub4 associates with the proteasome, we expressed Cim5-TAP in cells expressing 3Flag-tagged Ub4, Ub4N, and Ub4C. Western blots show that the full-length and truncated proteins were present in Cim5-TAP pull-down (Fig. 8C), indicating that both N- and C-terminal regions of Ub4 are involved in the interaction with the proteasome. Thus, Cdc48-Ub4 may act directly on the proteasome to promote its distribution in the nucleus.

DISCUSSION

Cdc48/VCP/p97 controls a variety of cellular processes through specific adaptors including the family of UBX domain proteins whose functions have not been well determined. Ub4 was previously shown to be involved in ERAD and degradation of several model substrates (9, 32). In this report we demonstrate that Cdc48 together with Ub4 are important for nuclear...
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FIGURE 8. Both UBL and UBX domains of Ubx4 are involved in the interaction with the proteasome. A, schematic representations of domain structures in Ubx4 and deletion mutants used in this study. B, both UBL and UBX domains are required for viability in cdc48-3. Wild-type, cdc48-3, and cdc48-3 PGAL-3HA-UBX4 cells carrying UBX4, Ubx4N, or Ubx4C as indicated were spotted in 10-fold serial dilutions from left to right on galactose or glucose plates. The plates were photographed after 2-3 days at room temperature. C, Ubx4 associates with the proteasome through both UBL and UBX domains. Ubx4, Ubx4N, or Ubx4C tagged with 3Flag was introduced into cdc48-3 PGAL-3HA-UBX4 cells with or without Cim5-TAP as indicated. TAP pull-down was performed, followed by Western blotting with anti-FLAG, anti-TAP, and anti-actin antibodies.

distribution of the 26S proteasome. Defects in this process perturb degradation of mitotic proteins Clb2 and Cdc20, leading to mitotic arrest.

We found that ubx4Δ is synthetic-lethal with cdc48-3 and that suppressing UBX4 expression in cdc48-3 cells has drastic effect on mitotic progression and proteasome distribution even at permissive temperature for cdc48-3. The lack of obvious phenotypes in ubx4Δ or in Ubx4-depleted cells suggests that another Cdc48 adaptor shares a similar function with Ubx4 and can support the function in the absence of Ubx4. The partially compromised activity of cdc48-3 at permissive temperature may work through both Ubx4 and the other redundant adaptor, causing only minor phenotypes of slight mitotic delay and small proteasome puncta in cdc48-3 cells. However, the other adaptor alone in cdc48-3 ubx4 double mutant may not be sufficient to maintain Cdc48 function, resulting in further compromised activity and enhanced phenotypes of large proteasome foci and mitotic arrest. This possibility predicts that deletion of both adaptors is likely lethal and would produce phenotypes similar to that of cdc48-3 ubx4. Previous finding shows that UBX4, UBX6, and UBX7 share redundant functions, since the triple deletion mutant is deficient in sporulation and degradation of model substrates (32). However, the triple mutant is viable under normal growth condition (32), suggesting that Ubx6 and Ubx7 are unlikely the redundant adaptors that share the function with Ubx4 in mitosis. Whether the putative redundant adaptor is another UBX domain protein or a novel molecule awaits future investigation.

We discover that proteasomes are mislocalized into foci at the inner side of the nuclear envelope in cdc48-3 ubx4 double mutant (Figs. 5 and 6). The foci likely contain the 26S proteasome, because subunits in both CP and RP form foci (Fig. 5, A–C). In mammalian cells, the inhibition of proteasome function or the overproduction of inefficiently folded or assembled proteins leads to the formation of centrosome-associated inclusions, termed aggresomes, that contain proteasome, ubiquitin, undegradable protein substrates and cytosolic chaperones (33). Similar structures have also been reported in budding yeast upon overexpression of human huntingtin protein with an expanded polyglutamine domain (34). However, inhibiting proteasome function by MG132 or mutations in the proteasome subunits do not cause proteasome aggregation in budding yeast (our unpublished results). Thus, the proteasome foci in cdc48-3 ubx4 cells are distinct from the aggresome in mammalian cells.

The 26S proteasome in cdc48-3 ubx4 cells is largely intact. By non-denaturing gel analysis of the proteasome, we demonstrate that CP can still assemble with its activator RP or Blm10 in cdc48-3 ubx4 cells and that the mutations have no apparent effect on the in-gel peptidase activity of the proteasome (Fig. 4). Interestingly, we found that ubx4Δ is synthetic-lethal with ump1Δ (Fig. 4D) and with rpn4Δ (Fig. 4E), indicating that optimal proteasome biogenesis is required for cell survival. However, the proteasome foci in cdc48-3 ubx4 double mutant are unlikely caused by impaired proteasome biogenesis, because we did not find a similar phenotype in cells with defective CP assembly due to the expression of C-terminal truncated Pre4 or HA-tagged Ump1.3

It has been shown that the proteasome is dynamically localized in budding yeast cells. It accumulates in the nucleus during exponential growth. At the transition from exponential to stationary phase, the proteasome is first enriched at the nuclear periphery, then forms immobile dots close to the nuclear periphery, and eventually accumulates as cytoplasmic storage granules (35). Interestingly, the proteasome foci in cdc48-3 ubx4 double mutant resemble in cells of early stationary phase, indicating that proteasomes may accumulate at the foci through a physiological process. Our FRAP analysis shows that proteasomes in the foci are turned over with a half-life of ~1.45 s, which is slower than that of nuclear proteasomes in normal cells (~0.66 s) (Fig. 5D). Thus, proteasomes are likely retained but not aggregated in the foci. Fission yeast proteasomes are

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enriched at the nuclear envelope via the nuclear envelope protein Cut8 that interacts with and tethers the proteasome (28). Cut8 homolog Sts1 in budding yeast is also important for nuclear accumulation of the proteasome (36). We discover that the proteasome foci in cdc48-3 ubx4 double mutants are colocalized with Sts1 (Fig. 6B), raising a possibility that the proteasome is retained at the nuclear envelope through Sts1. Because Cdc48 is known to function as a segregase to extract proteins, Cdc48-Ubx4 may facilitate the release of proteasomes from Sts1. The formation of proteasome and Sts1 foci by ATPase-defective Cdc48Q9Q mutant (Fig. 6D) indicates that Cdc48 may exert its chaperone activity during ATP hydrolysis to promote proteasome distribution.

cdc48-3 ubx4 double mutant is defective in the degradation of mitotic proteins Cib2 and Cdc20, causing a delay in anaphase. This defect is likely a result of proteasome clustering at the nuclear envelope that reduces the level of available proteasomes. This notion is supported by the finding that the growth and proteolytic defects of cdc48-3 ubx4 can be rescued by up-regulation of proteasome gene expression through Rpn4. Cib2 and Cdc20 seem to be more sensitive to proteasome mislocalization than several other short-lived proteins tested. Consistent with this notion, temperature sensitive sts1 was originally isolated as a metaphase mutant and named as dbf8 (for dumbbell former 8) (37). Similarly, mutations in cut8, the fission yeast homolog of STS1, also perturb anaphase and produce a “cut” phenotype (38). Together, these findings demonstrate that proteasome mislocalization impacts on mitosis and that efficient degradation of mitotic regulators requires an optimal level of the proteasome. Our study provides a further link between mitotic progression and proteasome homeostasis mediated by Cdc48-Ubx4.

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