The AAA-ATPase Cdc48 and cofactor Shp1 promote chromosome bi-orientation by balancing Aurora B activity

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Summary

The assembly, disassembly and dynamic movement of macromolecules are integral to cell physiology. The ubiquitin-selective chaperone Cdc48 (p97 in Metazoa), an AAA-ATPase, might facilitate such processes in the cell cycle. Cdc48 in budding yeast was initially isolated from a mitotic mutant. However, its function in mitosis remained elusive. Here we show that the temperature-sensitive cdc48-3 mutant and depletion of cofactor Shp1 (p47 in Metazoa) cause cell-cycle arrest at metaphase. The arrest is due to a defect in bipolar attachment of the kinetochore that activates the spindle checkpoint. Furthermore, Cdc48-Shp1 positively regulates Glc7/protein phosphatase 1 by facilitating nuclear localization of Glc7, whereas it opposes Ipl1/Aurora B kinase activity. Thus, we propose that Cdc48-Shp1 promotes nuclear accumulation of Glc7 to counteract Ipl1 activity. Our results identify Cdc48 and Shp1 as critical components that balance the kinase and phosphatase activities at the kinetochore in order to achieve stable bipolar attachment.

Key words: Kinetochore, Mitosis, Saccharomyces cerevisiae, AAA-ATPase, Cdc48

Introduction

Budding yeast Cdc48 and its metazoan homolog p97 (also known as valosin-containing protein; VCP), is an abundant and evolutionarily conserved protein. Cdc48/p97 belongs to the AAA (ATPase associated with a variety of cellular activities) ATPase superfamily that participates in diverse cellular processes. Cdc48/p97 is best known for its function in homotypic membrane fusion of organelles (Latterich et al., 1995; Rabouille et al., 1995), ER-associated protein degradation (ERAD) (Bar-Nun, 2005), and ubiquitin and/or proteasome-mediated protein degradation (Rumpf and Jentsch, 2006). Because these processes involve protein ubiquitinylation, Cdc48/p97 is considered as a ubiquitin-selective chaperone (Jentsch and Rumpf, 2007; Ye, 2006).

Cdc48/p97 exerts its diverse functions through an array of cofactors. Cdc48 coupled with the Npl4-Ufd1 complex participates in (1) mobilization of a ubiquitinylated transcription factor from the membrane for nuclear translocation (Rape et al., 2001), (2) extracting proteins from ER for degradation (ERAD) (Bays et al., 2001; Ye et al., 2001), and (3) nuclear expansion following nuclear envelope formation (Hetzer et al., 2001). Both Npl4 and Ufd1 contain a ubiquitin-binding domain that mediates the interaction with ubiquitylated substrates. In addition, the cofactor p47 links p97 to targets involved in membrane fusion (Kondo et al., 1997; Rabouille et al., 1998) and the formation of a closed nuclear envelope (Hetzer et al., 2001). p47 has a ubiquitin-associated (UBA) domain that can bind ubiquitin. It also associates with a deubiquitylating enzyme to facilitate Golgi membrane reassembly after mitosis (Wang et al., 2004). p47 interacts with p97 through a ubiquitin-related (UBX) domain that is structurally similar to ubiquitin (Yuan et al., 2001). Besides p47, other UBX-domain proteins bind Cdc48 and probably act as cofactors (Buchberger, 2002). In budding yeast, there are seven UBX domain proteins, Ubx1-7 (Schuberth et al., 2004). Ubx1, also known as Shp1 (Suppressor of high copy protein phosphatase 1), is most similar to mammalian p47 (Zhang et al., 1995). Both Shp1 and Ubx3 contain a UBA domain and serve as cofactors for Cdc48 in ubiquitin-dependent protein degradation (Hartmann-Petersen et al., 2004; Schuberth et al., 2004). Ubx4, Ubx6 and Ubx7 (also known as Cui1-3) also function in ubiquitin-mediated proteolysis and sporulation (Decottignies et al., 2004). Thus, each cofactor might confer multiple functions on Cdc48/p97.

Budding yeast Cdc48 was initially isolated from a cell cycle mutant that arrested in mitosis at the restrictive temperature (Moir et al., 1982). However, the function of Cdc48 in the cell cycle is not well understood. Cdc48/p97 itself is under cell cycle control. Budding yeast Cdc48 is mostly associated with the nuclear envelope and peripheral ER (Latterich et al., 1995), and enters the nucleus during late G1 (Madeo et al., 1998). Human p97 accumulates at the centrosome during mitosis (Madeo et al., 1998), p97 and its partner p47 are phosphorylated in vitro by the mitotic kinase Cdc2 (Mayr et al., 1999). Phosphorylation of p47 by Cdc2 is required for Golgi disassembly during mitosis (Uchiyama et al., 2003). It is not clear if the interaction between Cdc48/p97 and its cofactors is controlled during the cell cycle. Cdc48/p97 appears to have multiple functions in the cell cycle. In budding yeast, Cdc48 is important for passing Start, the cell cycle commitment point in G1, by degrading the G1-cyclin-dependent kinase inhibitor Far1 (Fu et al., 2003). In fission yeast, Cdc48 is required for the metaphase-to-anaphase transition by stabilizing Separase (Ikai and Yanagida, 2006), the enzyme that cleaves cohesin components to separate sister chromatids (Uhlmann et al., 1999). In addition, Cdc48/p97 together with Npl4-Ufd1 has been shown to be involved in spindle disassembly during mitotic exit in both budding yeast and X. egg extracts (Cao et al., 2003), although the results are controversial (Heubes and Stemmann, 2007).
Mitosis must be executed with a precise order to evenly segregate sister chromatids. A critical step in this process is the assembly of kinetochores at the centromere and binding of sister kinetochores to microtubules emanating from the opposite spindle poles. The attachment of sister kinetochores to microtubules from the same pole is corrected by Aurora B protein kinase (Ipl1 in budding yeast), that promotes turnover of microtubules from improperly attached kinetochores (Pinsky et al., 2006b; Tanaka et al., 2002). Once all kinetochores have established bipolar attachment, the cell can then enter anaphase to segregate sister chromatids. Anaphase is triggered by the E3 ubiquitin protein ligase Anaphase-promoting complex/cyclosome (APC/C) (Thornton and Toczyski, 2006). By binding to the activator-adaptor Cdc20, APC/C targets the anaphase inhibitor Securin (Pds1 in budding yeast) for degradation, leading to cleavage of the cohesin complex and separation of sister chromatids (Yu, 2007). Later in anaphase, APC/C is coupled with another adaptor Cdh1 to target the degradation of Cdc20 and mitotic cyclins for mitotic exit (Huang et al., 2001; Schwab et al., 2001). The presence of any unattached kinetochore triggers the spindle checkpoint to inhibit APC/C-Cdc20, thus blocking anaphase onset (Burke and Stukenberg, 2008). In addition, budding yeast at metaphase normally aligns the spindle along the mother-bud axis with the nucleus positioned at the bud neck, so that the nucleus divides into both the mother and the bud after anaphase. A mis-oriented spindle triggers the spindle position checkpoint, composed of the checkpoint proteins Bub2 and Bfa1, to inhibit APC/C-Cdh1, thus blocking mitotic exit (Fraschini et al., 2008).

To better understand the function of Cdc48/p97 in the cell cycle, we examined the phenotypes of the cdc48-3 mutant in budding yeast. We show that the mutant arrests at metaphase as a result of defects in kinetochore bi-orientation. By screening the known cofactors of Cdc48, we show that Shp1 mediates the kinetochore function of Cdc48. Furthermore, we present evidences that Cdc48-Shp1 facilitates nuclear accumulation of Glc7/Protein phosphatase 1 to counteract Ipl1 activity, allowing kinetochores to establish stable bipolar attachment to the spindle.

Results

The cdc48-3 mutant arrests at metaphase

In order to understand the cell cycle function of Cdc48, we first examined the phenotypes of the temperature-sensitive cdc48-3 mutant that was previously shown to be defective in ER membrane fusion (Latterich et al., 1995) and spindle disassembly (Cao et al., 2003). After 4 hours at 38.5°C, cdc48-3 cells were arrested with a large bud. Immunofluorescence staining of tubulin showed that the large-budded cdc48-3 cells mostly contained a short spindle (Fig. 1A), indicative of metaphase arrest. Normal metaphase cell aligns the spindle along the mother-bud axis and position the nucleus near the bud neck (Fig. 1Aa-c). However, ~47% of large-budded cdc48-3 cells contained a spindle that was not aligned along the cell axis, even though the astral microtubules appeared normal (Fig. 1Ag-i, B). Consequently, the nucleus in cdc48-3 cells was often displaced from the bud neck (Fig. 1A). Metaphase arrest was also observed at 37°C, although spindle mis-orientation was more prominent at 38.5°C than at 37°C (data not shown). In this study, we focus on the metaphase function of Cdc48. The control of spindle orientation by Cdc48 will be reported elsewhere.

To further confirm that at the restrictive temperature the cdc48-3 mutant arrests at metaphase, we performed immunoblot analysis to examine the anaphase inhibitor Pds1/Securin. The protein accumulates from S to M phase and is degraded at the metaphase-to-anaphase transition (Cohen-Fix et al., 1996). We analyzed Pds1 levels during cell cycle entry after α-factor withdrawal. The experiment was performed at 37°C instead of 38.5°C because G1 progression was much delayed in cdc48-3 cells at 38.5°C (our unpublished result). Immunoblots showed that the level of Pds1 fluctuated in wild-type cells during cell cycle progression (Fig. 1C). cdc48-3 cells also accumulated Pds1 during cell cycle entry. However, the protein persisted for at least 4 hours after α-factor withdrawal.
cdc48-3 activates the spindle checkpoint
We next investigated whether the metaphase arrest of cdc48-3 cells is dependent on the spindle checkpoint that blocks anaphase onset in response to unattached kinetochores (Burke and Stukenberg, 2008). We deleted the evolutionarily conserved spindle checkpoint gene MAD2 and examined the phenotypes of cdc48-3 mad2Δ mutant. At the restrictive temperature, the fraction of the double mutant with a metaphase spindle was significantly reduced and cells with an anaphase spindle increased, compared with that of the cdc48-3 mutant (Fig. 1B). Interestingly, ~20% of the double mutant contained an unevenly divided DNA mass (Fig. 1A,B), indicating that perturbation of the spindle checkpoint in cdc48-3 mutants led to aberrant anaphase with chromosome mis-segregation. Consistent with the increase of anaphase cells, immunoblots showed that the level of Pds1 was no longer maintained in cdc48-3 mad2Δ during a synchronized cell cycle (Fig. 1C). These results suggest that metaphase arrest of cdc48-3 cells requires the spindle checkpoint.

cdc48-3 causes defective kinetochore-microtubule attachment
Activation of the spindle checkpoint in cdc48-3 mutants indicates a defect in attachment of kinetochores to the mitotic spindle, which is already supported by the finding that DNA was unevenly segregated when the checkpoint was disabled in cdc48-3 cells (Fig. 1A,B). To further confirm this notion, we visualized chromosome III by GFP tagging as described previously (Straight et al., 1997). Briefly, a tandem array of 256 copies of Lac operator sequence (LacO) was integrated at ~23 kb from the centromere of chromosome III in cells expressing GFP-Lac repressor (LacI). Binding of GFP-LacI to LacO marks the position of chromosome III. The cells also expressed Spc42-mCherry to visualize the spindle pole body (SPB). In normal metaphase cells, the GFP signal of chromosome III was mostly in between the SPBs (Fig. 2A). In anaphase, there were two GFP-chromosome III signals, and each was associated with a SPB (Fig. 2A). However, in ~35% of large-budded cdc48-3 cells GFP-chromosome III associated closely with a single SPB, instead of in between the poles (Fig. 2A,B). In the small fraction of cdc48-3 cells that were in anaphase, GFP-chromosome III sometimes associated with only one SPB or strayed away from the SPB (Fig. 2A,B). These results suggest that the cdc48-3 mutant is defective in kinetochore attachment to the spindle, leading to chromosome mis-segregation in anaphase. In addition, we consistently observed three or more Spc42-mCherry signals in a small fraction of large-budded cdc48-3 cells (Fig. 2B). Furthermore, ~6% of large-budded cdc48-3 contained only one Spc42-mCherry signal, instead of two, suggesting that the cdc48-3 mutation might also affect duplication, separation or maintenance of the SPB.

Notably, the distance between the Spc42-mCherry signals in cdc48-3 cells was larger than in wild-type cells at metaphase (Fig. 2A). The pole-to-pole distance was normally 0.5-2.0 μm in most of the pre-anaphase cells, whereas the distance increased to 1-3 μm in cdc48-3 mutants (Fig. 2C). The length of the spindle is controlled by the force that separates the spindle poles and the force that pulls the poles together through linked sister kinetochores (Saunders et al., 1997). A defect in chromosome cohesion or kinetochore-microtubule attachment results in an elongated spindle at metaphase, because of imbalanced forces, as seen in the kinetochore mutants mis6 and mis12 in fission yeast (Goshima et al., 1999) and in the budding yeast ip11 mutant (Biggins et al., 1999). The increased distance between the SPBs in cdc48-3 is consistent with a defect in kinetochore-microtubule attachment.
Cofactor Shp1 is required for mitotic progression

Cdc48/p97 exerts its diverse functions through specific cofactors. We reason that one or more of these cofactors might mediate the mitotic functions of Cdc48 and their loss-of-function mutations probably cause similar phenotypes to that of cdc48-3. We thus examined the temperature-sensitive npl4-1 and ufd1-2 mutants and the deletions of UBX2-7 genes, but did not find clear cell cycle phenotypes in these mutants. Because we were unable to delete SHP1 (UBX1) in our strain background (W303), we generated PGAL-3HA-SHP1 by inserting a galactose-inducible promoter and a 3HA tag upstream of the endogenous SHP1 gene to conditionally control its expression. Immunoblot analysis showed that 3HA-Shp1 was largely depleted within 30 minutes after changing the growth medium from galactose (YPGal) to glucose (YPD; Fig. 3A). PGAL-3HA-SHP1 cells grew on YPGal, but not on YPD (Fig. 3B), indicating that SHP1 is essential for viability in our strain. Even though 3HA-Shp1 expression was quickly repressed in YPD, the growth rate did not decline for 6 hours (data not shown), probably because of residual expression from the GAL promoter. Fluorescence-activated cell sorter (FACS) analysis showed that PGAL-3HA-SHP1 cells gradually arrested with 2N DNA content after 9 hours in YPD (Fig. 3D). Immunofluorescence staining of tubulin showed that most of the large-budded PGAL-3HA-SHP1 cells contained a short spindle (Fig. 3C). These results indicate that Shp1 depletion delays the cell cycle at metaphase. Furthermore, Pds1 accumulated and was sustained in Shp1-depleted cells after release from α-factor arrest in YPD (Fig. 3E), consistent with metaphase arrest. Similar to cdc48-3, the metaphase arrest of Shp1-depleted cells requires a functional spindle checkpoint, because Pds1 in the PGAL-3HA-SHP1 mad2Δ strain degraded with kinetics similar to that in wild-type cells (Fig. 3E). FACS analysis also showed that PGAL-3HA-SHP1 mad2Δ cells did not arrest with 2N DNA content in YPD (Fig. 3D). These results demonstrate that Shp1 depletion triggers the spindle checkpoint, leading to metaphase arrest.

Shp1 depletion causes defects in kinetochore attachment

We next determined whether Shp1 depletion affected kinetochore-microtubule attachment, as in cdc48-3 cells. By examining GFP-chromosome III and Spc42-mCherry, we found that GFP-chromosome III associated closely with one of the Spc42-mCherry signals in ~25% of large-budded PGAL-3HA-SHP1 cells after 12 hours in YPD (Fig. 4A,B). In cells that had separated GFP-chromosomes III (~33%), the signals were largely segregated into both the mother and the bud, and a small fraction (<1%) of the large-budded cells contained GFP-chromosome III signals only in the mother cell (Fig. 4A,B), indicating that some of the cells were able to correctly attach the kinetochore and enter anaphase. In addition, ~7% of the large-budded cells contained an aberrant number of SPBs (one or more than two; Fig. 4B), suggesting that Shp1 depletion might also affect duplication, separation or integrity of the SPB. Therefore, the mitotic phenotypes caused by Shp1 depletion were similar to those of the cdc48-3 mutation, although the phenotypes were generally less severe than those in cdc48-3 cells. In addition, the pole-to-pole distance in Shp1-depleted cells was similar to that in wild type (Fig. 4C), whereas the distance was greater in cdc48-3 cells (Fig. 2C), consistent with the notion that the kinetochore attachment defect in Shp1-depleted cells was less severe than that in cdc48-3 cells. Furthermore, Mad1 is phosphorylated upon spindle checkpoint activation (Hardwick and Murray, 1995). We found that Mad1 in cdc48-3 was phosphorylated to a lesser extent than that in wild-type cells arrested at metaphase with nocodazole (supplementary material Fig. S1), which might reflect the degree of spindle checkpoint activation. The checkpoint was maximally activated by complete detachment of kinetochores upon nocodazole treatment, whereas only some kinetochores were unattached in cdc48-3. Mad1 mobility shift was hardly detected in Shp1-depleted cells (supplementary material Fig. S1), consistent with a less severe defect in Shp1-depleted cells. It is possible that repression of Shp1 expression from the GAL promoter in glucose medium was not complete, so that residual Shp1 was able to support some function.

We next examined whether cdc48-3 and Shp1-depleted cells are defective in bipolar attachment of the kinetochore. In pre-anaphase

Fig. 3. Shp1 depletion causes metaphase delay. (A) PGAL-3HA-SHP1 was first grown in YPGal. The medium was then changed to YPD to repress the expression of 3HA-Shp1, and samples were taken at the indicated times for western blots of 3HA-Shp1 and Mad2. Mad2 served as the loading control. (B) SHP1 and PGAL-3HA-SHP1 cells were plated on YPGal (Gal) or YPD (Glu) medium. The plates were photographed after 2-3 days at 30°C. (C) SHP1 and PGAL-3HA-SHP1 cells were grown in YPD for 12 hours and processed for immunofluorescence staining of tubulin. DNA was stained with DAPI. Scale bar, 5 μm. (D) DNA contents were measured in cells that were first grown in YPGal and then shifted to YPD for the indicated times. (E) Western blots of Pds1-8myc and Mad1. Cells were first grown in YPGal to mid-log phase at 30°C. The medium was then changed to YPD for 4 hours to repress Shp1 expression before the addition of α-factor. After 4 hours, the cells were released from the arrest in YPD, and samples were taken for western blotting.
cells, sister kinetochores and the centromere proximal region are normally split by the force generated from bipolar attachment to the spindle (Goshima and Yanagida, 2000; He et al., 2000; Pearson et al., 2001; Tanaka et al., 2000). To visualize the centromere, we tagged chromosome III with GFP-LacI by integrating LacO repeats at ~1.3 kb from CEN3. We obtained metaphase cells by repressing the expression of CDC20 from the GAL promoter. As expected, two GFP-CEN3 signals were present between Spc42-mCherry dots in these cells (Fig. 5A), consistent with bi-orientation of the kinetochore. However, the CEN3-GFP signal was not split in ~20% of cdc48-3 cells at 37°C (Fig. 5A,B), indicating that the kinetochrome was mono-oriented or unattached. Similarly, ~24% of Shp1-depleted cells at metaphase contained only one GFP-chromosome III signal.

Cdc48-Shp1 facilitates nuclear accumulation of Glc7

Budding yeast Shp1 (Suppressor of high copy PP1) was initially isolated as a regulator of the type I protein phosphatase (PP1) (Zhang et al., 1995). The catalytic subunit of PP1 in budding yeast is encoded by the GLC7 gene that is important for many processes and is essential for viability. Glc7 localizes primarily in the nucleus and the bud neck during the cell cycle (Bloecher and Tatchell, 2000). It also accumulates at the SPB from anaphase to cytokinesis, and then at the cytokinesis ring (Bloecher and Tatchell, 2000).

Although Glc7 is not found at the kinetochore, certain conditional glc7 mutants are blocked at metaphase at the restrictive temperature (Hisamoto et al., 1994; MacKelvie et al., 1995). Similar to cdc48-3 and Shp1-depleted cells, glc7 mutants are defective in the kinetochore-microtubule attachment that activates the spindle checkpoint (Bloecher and Tatchell, 1999; Sassoon et al., 1999). Because a mutation in SHP1 suppresses the lethality caused by a high copy of GLC7 (Zhang et al., 1995), it is possible that Cdc48-Shp1 acts to promote the activity of Glc7 towards its targets at the kinetochores. To test this hypothesis, we examined the genetic interaction between cdc48-3 and glc7-12 that, at the restrictive temperature, exhibit mitotic phenotypes (MacKelvie et al., 1995). Both cdc48-3 and glc7-12 showed slow growth at 30°C and almost no growth at 33°C, whereas the cdc48-3 glc7-12 double mutant grew poorly even at 25°C (Fig. 6A), suggesting that Cdc48 and Glc7 act in the same cellular process. In addition, overexpression of Glc7 from the galactose-inducible promoter perturbed cell growth (Fig. 6B), whereas the growth defect of PGAL-GLC7-4HA can be partially suppressed by cdc48-3 (Fig. 6B). This result is similar to the previous finding that the shp1 mutation suppresses the lethality of high copy GLC7 (Zhang et al., 1995) These genetic data are consistent with our model that Cdc48-Shp1 promotes the function of Glc7. To determine the effect of Cdc48-Shp1, we examined the localization of Glc7 in cdc48-3 and Shp1-depleted cells. Consistent with previous reports, GFP-Glc7 expressed from its own promoter localized predominantly in the nucleus (Fig. 6C). However, GFP-Glc7 signal was significantly reduced in the nucleus of cdc48-3 cells at 37°C and in Shp1-depleted cells (Fig. 6C), indicating that both Cdc48 and Shp1 are required for nuclear accumulation of Glc7.

Cdc48-Shp1 counteracts Ipl1 activity

Genetic and biochemical studies have shown that Glc7 opposes the kinase activity of Ipl1 (Francisco et al., 1994; Hsu et al., 2000; Pinsky et al., 2006a). IPL1 encodes Aurora B kinase that is known to promote turnover of microtubules from improperly attached kinetochores and is important for bi-orientation of chromosomes (Pinsky et al., 2006b; Tanaka et al., 2002). We thus examined the genetic interaction between IPL1 and CDC48. Both ipl1-321 and cdc48-3 mutants grew slower than wild-type cells at 30°C, and did not grow at 33°C (Fig. 7A). The ipl1-321 cdc48-3 double mutant still grew slowly at 30°C (Fig. 7A), suggesting that ipl1-321 and cdc48-3 did not complement each other. This result might indicate that Cdc48 did not oppose the function of Ipl1. However, it is possible that cdc48-3 has other defects that cannot be rescued by reduced Aurora B activity of ipl1-321. We further tested the genetic
interaction between \textit{IPL1} and \textit{SHP1}. Both \textit{P\textsubscript{GAL}}-3HA-SHP1 and \textit{ipl1-321 P\textsubscript{GAL}}-3HA-SHP1 cells did not grow on YPD plates at 25°C (Fig. 7B), whereas the growth was partially restored in \textit{ipl1-321 P\textsubscript{GAL}}-3HA-SHP1 at 30°C and 33°C (Fig. 7B). This result is consistent with the notion that reduced nuclear Glc7 upon depletion of Shp1 can complement the defect of reduced Aurora B activity in \textit{ipl1-321} cells.

It has been shown that Ipl1 phosphorylates Dam1 (Kang et al., 2001; Li et al., 2002), a component of the Dam1 complex that is involved in kinetochore-spindle attachment and chromosome segregation (Cheeseman et al., 2001; Westermann et al., 2005). Glc7 has been shown to dephosphorylate Ipl1 targets, including Dam1 and histone H3 (Hsu et al., 2000; Pinsky et al., 2006a), and the balance of kinase and phosphatase activities is important for proper kinetochore attachment (Francisco et al., 1994; Sassoon et al., 1999). We thus examined the effect of the \textit{cdc48-3} mutation and Shp1 depletion on Dam1 phosphorylation. For detection by western blot, Dam1 was tagged by 8myc at the C-terminus (supplementary material Fig. S2). As reported previously, Dam1 phosphorylation was greatly reduced in \textit{ipl1-321} cells at 33°C (Fig. 7C), whereas the phosphorylation was not affected in \textit{cdc48-3} or \textit{P\textsubscript{GAL}}-3HA-SHP1 cells in YPD (Fig. 7C). Interestingly, Dam1 phosphorylation was restored in the \textit{ipl1-321 cdc48-3} double mutant at 33°C and in \textit{ipl1-321 P\textsubscript{GAL}}-3HA-SHP1 in YPD (Fig. 7C), consistent with the notion that Cdc48-Shp1 opposes Ipl1 activity. Therefore, we conclude that Cdc48-Shp1 is required to balance Ipl1 activity at the kinetochore.

**Discussion**

Our study uncovers a new function for Cdc48 in kinetochore bi-orientation during mitosis, and we demonstrate that the kinetochore function of Cdc48 is mediated by the cofactor Shp1. Both \textit{cdc48-3}-mutant and Shp1-depleted cells are delayed at metaphase because of a defect in kinetochore-microtubule attachment that activates the spindle checkpoint. By live-cell microscopy, we show that \textit{cdc48-3}-mutant and Shp1-depleted cells are defective in bi-orientation because of a decrease in \textit{ipl1/Aurora B} kinase activity (supplementary material Fig. S3). Instead, Cdc48 and Shp1 are required for nuclear accumulation of Glc7/PP1 to counteract Ipl1/Aurora B activity. Because Ipl1 promotes microtubule turnover at kinetochores, Glc7 probably dephosphorylates the target of Ipl1 at the kinetochore in order to stabilize kinetochore-microtubule attachment once bi-orientation is achieved (Fig. 7D). In fact, it has been shown that the kinetochore-microtubule binding activity in vitro is low in extracts prepared from \textit{glc7-10} mutants and from cells treated with...
phosphatase inhibitors (Sassoon et al., 1999). Thus, the reduced level of nuclear Glc7 in cdc48-3 or Shp1-depleted cells probably weakens kinetochore-microtubule interaction, resulting in an increase of unattached kinetochores that activate the spindle checkpoint. However, we cannot exclude the possibility that Cdc48 and Shp1 might also be involved in the assembly of functional kinetochores.

It has been shown that the cdc48-3 mutant is defective in spindle disassembly during mitotic exit (Cao et al., 2003). By introducing the cdc48-3 gene into a new strain, we now clearly demonstrate that this mutant is delayed at metaphase, instead of mitotic exit. We have noticed that cdc48-3 cells accumulate suppressor activities during prolonged culturing. Thus, we suspect that the different terminal phenotypes in our study and the earlier work might be attributable to some difference in the strain backgrounds or growth conditions.

Although cdc48-3 and Shp1-depleted cells share mitotic phenotypes, there is also a notable difference. The mitotic spindle is often improperly positioned in cdc48-3 cells, but not in Shp1-depleted cells. Normally, the spindle is oriented early in the cell cycle by the motor protein Myo2 that moves cytoplasmic microtubules along polarized actin cables into the bud (Segal and Bloom, 2001). It is possible that Cdc48 uses another cofactor in the cytoplasm, rather than Shp1, to control spindle orientation.

In addition to chromosome bi-orientation, Cdc48 appears to have other functions in mitosis. We have consistently observed an abnormal number of SPBs (one or three) in a small subset of large-budded cdc48-3 and Shp1-depleted cells, indicating that Cdc48 and Shp1 might also regulate the duplication, separation or maintenance of the SPB. It has also been shown that the cold-sensitive cdc48-1 mutant arrests as large-budded cells with unseparated SPBs (Frohlich et al., 1991). The cdc48-1 mutant appears to have a more severe defect in the SPB than cdc48-3 does. Human p97 is found at the centrosome in mitosis (Madeo et al., 1998), consistent with a role at the spindle pole. Interestingly, it has been previously shown that the SPB duplication phenotypes of mutations in the SPB components MPS2 and NDC1 can be suppressed by disruption of ERAD components (McBratney and...
Cdc48 is best known for its functions in membrane fusion, ERAD and protein degradation. Emerging evidences suggest that Cdc48 might exert these diverse functions through ubiquitinated substrates and act as a ubiquitin-selective chaperone (Ye, 2006). It is possible that Cdc48 also targets a ubiquitinylated protein in order to promote nuclear accumulation of Glc7. The cofactor Shp1 has a ubiquitin-associated domain (UBA) that can bind ubiquitin and mediate the interaction with the target. Glc7 is known to act on specific substrates by association with a range of regulatory or targeting subunits (Tu et al., 1996). It remains to be determined whether Glc7 or its regulatory subunit is ubiquitinated and recognized by Cdc48-Shp1. Identifying the direct target of Cdc48-Shp1 is crucial for understanding how Cdc48-Shp1 might use its chaperone activity to facilitate the assembly of Glc7 with the regulatory subunit or to promote the nuclear import or nuclear retention of Glc7 holoenzyme.

The kinetochore function of Cdc48 is not unique to yeast. It has been shown that RNAi of p97 in human HeLa cells causes abnormal mitosis, with a disordered spindle and mis-aligned chromosomes (Wojcik et al., 2004), suggesting that p97 is also important for kinetochore function in metazoans. The cofactor for p97 in spindle organization and kinetochore function remains to be identified. Budding yeast Shpl is most similar to human p47, with 30% identity and 51% similarity. In animal systems, the p97-p47 complex is known to function in post-mitotic membrane fusion and nuclear envelope formation (Hetzer et al., 2001; Rabouille et al., 1995). It remains to be determined whether the p97-p47 complex has additional functions in chromosome bi-orientation. In budding yeast, we demonstrate that Cdc48-Shp1 is important for kinetochore bi-orientation by promoting nuclear accumulation of PP1 to counteract Aurora B activity. However, the nuclear envelope of metazoan cells is dissolved during mitosis. Thus, p97 might control chromosome alignment by different mechanisms. Interestingly, Cdc48/p97 has also been shown to inhibit Aurora B in metazoans. In Xenopus egg extracts, p97 has been shown to bind ubiquitinylated Aurora B and extract the protein from chromatin in order for the nuclear envelope to assemble after mitosis (Ramadan et al., 2007). In addition, a Cdc48 homolog directly binds Aurora B and inhibits its kinase activity from metaphase to telophase in C. elegans (Heallen et al., 2008). Together with our results, these studies show that Cdc48/p97 controls Aurora B activity at multiple levels during the cell cycle.

Materials and Methods

Construction of yeast strains

All strains used in this study are given in Supplementary material Table S1. To obtain cdc48-3 in the W303 strain background, the cdc48-3 strain (MYL1640) was backcrossed four times to W303 cells. Alternatively, the cdc48-3 gene was amplified by PCR from MLY1640, including ~600 bp of the upstream and ~1.2 kb of the downstream sequences. The selection marker HIS3 was inserted into the downstream sequence. The construct was then transformed into W303 cells to replace endogenous CDC48 by homologous recombination. The cell cycle phenotypes of cdc48-3 cells obtained from both approaches were essentially the same. To tag Pds1, four copies of HA or eight copies of myc sequence were inserted before the Stop codon of PDS1 in pRS405 vector that contained the entire PDS1 coding sequence and 1 kb of the downstream region. The plasmid was cut with BglII within the coding region for integration at the genomic locus. GFP-tagging of chromosome III was performed by integrating plasmid pAFLS59 carrying 256 LacO repeats at the LEU2 locus as described previously (Straight et al., 1997). For GFP-tagging of CEN3, the putative open reading frame YCL001W-A with 400 bp downstream region was cloned into pAFLS9 at the KpnI and SacI sites. The plasmid (pRC1558) was cut with Nhel and integrated at the YCL001W-A locus. Spec42-mCherry and Ipl1-13myc were generated by PCR-mediated integration (Longtine et al., 1998). pGL-3HA-Shp1 was also generated by PCR-mediated integration using plasmid pFAba-kanMX6-pGAL-3HA as the template in PCR (Longtine et al., 1998). mut2A was generated by transformation with the plasmid pRC01.1 containing upstream and downstream sequences of MAD2 with the coding region replaced by the selection marker URA3. For GFP-Glc7, we generated plasmid pRC1580 that contains the coding region of GLC7 downstream of the GFP sequence and 500 bp of the GLC7 promoter region before GFP in pRS316. The plasmid was linearized with Sall and integrated at the URA3 locus. For Dam1-8myc, eight copies of the myc sequence were inserted before the Stop codon of DAM1 in pRS404 or pRS405 vectors containing the entire DAM1 coding sequence and ~400 bp of the downstream region. The plasmid was cut with BglII within the coding region for integration at the genomic locus.

Yeast cell lysates and western blots

For western blots, 1.5 ml of cell culture at OD600 between 0.5 and 1.0 were collected. Cells were cooled on ice and washed once with cold TE [10 mM Tris (pH 8.0), 1 mM EDTA]. Cell lysates were prepared in lysis buffer [10 mM KPO4 (pH 7.2), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM Na3VO4, 1 mM sodium vanadate, 0.5% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and 10 µg/ml each of leupeptin, pepstatin and chymostatin] by mixing the cell pellets with an equal volume of Zirconia beads and beating for 1.5 minutes at 4°C in a mini-headbeater (Biospec Products). Samples were centrifuged at 21, 130 g for 5 minutes at 4°C, and the supernatants were used as the cell lysates. Protein concentrations were determined using a protein assay kit (Bio-Rad) and adjusted to the same concentrations with lysis buffer. 10-30 µg of total proteins in the lysates were resolved by SDS-PAGE and proteins were transferred to nitrocellulose membranes for western blotting with anti-HA (16B12; Covance), anti-Myc (A14; Santa Cruz), anti-Mad1 (Hardwick et al., 1995) and anti-Mad2 antibodies (Chen et al., 1999).

Immunofluorescence staining

Yeast cells were fixed in 3.7% formaldehyde at the last growth temperature for 5 minutes and then at room temperature for 2 hours. After treatment with Zymolyase (Seikagaku Corp.) to remove the cell wall, the spheroplasts were attached to multi-well slides coated with poly-lysine, followed by incubation in methanol for 6 minutes and then in acetone for 30 seconds at −20°C. The samples were then blocked with PBS containing 1 mg/ml BSA. Microtubules were stained with a mouse anti-tubulin antibody (Molecular Probes) in the blocking solution, and then detected with Texas Red-conjugated goat anti-mouse antibody (Molecular Probes). DNA was stained with 1 µg/ml DAPI.

Microscopy

Immunofluorescence images were taken using a ×100 1.4 numerical aperture lens and a charged-coupled device (CCD) camera (MicroMAX-5 MHz; Princeton Instruments) on a Zeiss Axiocam2 fluorescence microscope. Images were collected and processed with Metamorph Imaging System (v6.2; Universal Imaging Corp.) and converted to Adobe Photoshop format. For live-cell imaging, cells were maintained in synthetic complete medium and then immobilized on a coverslip coated with 20 µg/ml of concanavalin A (Sigma). The coverslip was then mounted on a slide and sealed with vacuum grease. Images were taken using a ×100 1.4 numerical aperture lens and an electron-multiplying CCD (EMCCD) camera (Cascade II 512; Photometrics) on an Olympus IX71 fluorescence microscope that was controlled by the DeltaVision system (Applied Precision). Sixteen optical sections with spacing of 0.5 µm were collected. Deconvolution and maximal projection of the images were performed by Softworx software. For time-lapse images, 10 optical sections with spacing of 0.6 µm were taken and similarly processed. The distances between objects on the images were measured by Softworx software.
Ipl1 kinase assay

Cellly Ly was prepared from 10 ml of cells at log phase by bead-beating in lysis buffer. For immunoprecipitation of Ipl1-31mye, 2 μl of anti-myc antibody (9E10) was incubated with 600 μg of total proteins in the lysates for 1 hour at 4°C, followed by incubation with 20 μl protein A beads that were pre-blocked by 1 mg/ml BSA. After 2 hours, the beads were washed four times with 1 ml lysis buffer and once with kinase buffer without ATP (50 mM Tris, pH 7.0, 1 mM DTT, 25 mM β-glycerophosphate, 5 mM MgCl2), and then resuspended in kinase buffer containing 10 μM ATP, 5 μM [γ-32P]ATP and 5 μg histone H3 for 30 minutes at 30°C. SDS-PAGE sample buffer was added to the beads to stop the reaction and samples were resolved by 15% SDS-PAGE for autoradiography.

This study was supported by grants from Academia Sinica and the National Science Council, Taiwan. We are grateful to Martin Latterich for cdc-69-1, Stefan Jennew and utf-1-2. Michael Stark for glc7-12, Andrew Murray for chromosome-GFP-tagging reagents, Keven Hardwick for anti-Mad1 antibody, Chao-Wen Wang for scientific input, and Sue-Ping Lee for technical assistance with imaging.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/12/2025/DC1

References


Supplementary Figure 1

**Supplementary Figure 1**

**Figure Description:**

The figure illustrates the effect of nocodazole treatment on Mad1 levels in various genetic backgrounds. The table lists different genetic strains and their responses to nocodazole treatment:

- **CDC48**: Wild-type CDC48
- **CDC48 deletion**: CDC48 deletion
- **cdc48-3**: CDC48 3rd allele
- **cdc48-3 mad2Δ**: CDC48 3rd allele with MAD2 deletion
- **SHP1**: Wild-type SHP1
- **SHP1 deletion**: SHP1 deletion
- **P_GAL-SHP1**: Inducible SHP1
- **P_GAL-SHP1 mad2Δ**: Inducible SHP1 with MAD2 deletion

The treatment conditions are indicated by:
- **-**: Nocodazole absent
- **+**: Nocodazole present

The Mad1 protein expression levels are shown for each condition, with samples labeled as follows:

1. **CDC48**
2. **CDC48 deletion**
3. **cdc48-3**
4. **cdc48-3 mad2Δ**
5. **SHP1**
6. **SHP1 deletion**
7. **P_GAL-SHP1**
8. **P_GAL-SHP1 mad2Δ**

*Note the asterisk (*) indicating a significant difference in Mad1 expression level.*
Supplementary Figure 3

The figure shows the effects of different combinations of the proteins CDC48, cdcl48-3, SHP1, P\textsubscript{GAL}-SHP1, and SHP1 on the expression of the protein IPL1-13myc. The expression levels of p-H3 and H3 are indicated at the bottom of the figure with bands for samples 1 to 5.
Table I. Yeast strains used in this study

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<th>Strain</th>
<th>Genotype</th>
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<td>cdc48-3 leu-2-3, 112 pep4Δ::URA3</td>
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RHC1826  

bar1Δ IPL1-13myc:TRP1 KanMX6:PGAL-3HA-SHP1

All strains, except MLY1640, are derivatives of W303 (ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1, MATa).