Regulation of Sli15/INCENP, kinetochore, and Cdc14 phosphatase functions by the ribosome biogenesis protein Utp7

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The Sli15–Ipl1–Bir1 chromosomal passenger complex is essential for proper kinetochore–microtubule attachment and spindle stability in the budding yeast *Saccharomyces cerevisiae*. During early anaphase, release of the Cdc14 protein phosphatase from the nucleolus leads to the dephosphorylation of Sli15 and redistribution of this complex from kinetochores to the spindle. We show here that the predominantly nucleolar ribosome biogenesis protein Utp7 is also present at kinetochores and is required for normal organization of kinetochore proteins and proper chromosome segregation. Utp7 associates with and regulates the localization of Sli15 and Cdc14. Before anaphase onset, it prevents the premature nucleolar release of Cdc14 and the premature concentration of Sli15 on the spindle. Furthermore, Utp7 can regulate the localization and phosphorylation status of Sli15 independent of its effect on Cdc14 function. Thus, Utp7 is a multifunctional protein that plays essential roles in the vital cellular processes of ribosome biogenesis, chromosome segregation, and cell cycle control.

Introduction

Ipl1 of the budding yeast *Saccharomyces cerevisiae* is the founding member of the Aurora family of protein kinases, and it is essential for proper chromosome segregation during mitosis and meiosis (Chan and Botstein, 1993; Francisco et al., 1994; Monje-Casas et al., 2007). It functions in a complex with Sli15 and Bir1 (Kim et al., 1999; Cheeseman et al., 2002), with Sli15 serving as a stimulator and targeting partner of Ipl1 (Kang et al., 2001). A mammalian homologue of Ipl1 (Aurora-B) also exists in a similar complex (termed the chromosomal passenger complex) that contains homologues of Sli15 (INCENP), Bir1 (Survivin), and the protein Borealin (Ruchaud et al., 2007). The Sli15–Ipl1–Bir1 complex regulates diverse processes during mitotic M phase, including spindle assembly (Kotwaliwale et al., 2007), kinetochore–microtubule attachment, and bi-orientation (Biggins et al., 1999; He et al., 2001; Tanaka et al., 2002; Dewar et al., 2004; Sandall et al., 2006); spindle assembly checkpoint activation in response to lack of kinetochore tension (Pinsky et al., 2006; King et al., 2007); anaphase spindle stabilization and elongation (Buvelot et al., 2003; Pereira and Schiebel, 2003; Bouck and Bloom, 2005; Higuchi and Uhlmann, 2005; Widlund et al., 2006); condensation and complete segregation of the ribosomal DNA locus in late anaphase (Lavoie et al., 2004; Sullivan et al., 2004); and coordination of cytokinesis to the clearance of chromosomes from the spindle midzone (Norden et al., 2006).

The Sli15–Ipl1–Bir1 complex undergoes changes in its subcellular localization to accomplish its many functions through M phase (He et al., 2001; Tanaka et al., 2002; Buvelot et al., 2003). Before anaphase onset, the Sli15 complex is concentrated mostly at kinetochores. Upon anaphase onset, this complex redistributes from kinetochores to the anaphase spindle. The redistribution of the Sli15 complex from kinetochores to the anaphase spindle requires dephosphorylation of Sli15 by the Cdc14 protein phosphatase, and ectopic activation of Cdc14 is sufficient for the redistribution of the Sli15 complex (Pereira and Schiebel, 2003).

The nucleolus is the site of ribosomal RNA (rRNA) transcription and processing. The small subunit (SSU) processome (or 90S preribosome), with ≥40 different protein subunits, processes the 35S pre-rRNA to generate the mature 18S rRNA that

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; HU, hydroxyurea; MEN, mitotic exit network; rRNA, ribosomal RNA; SSU, small subunit.

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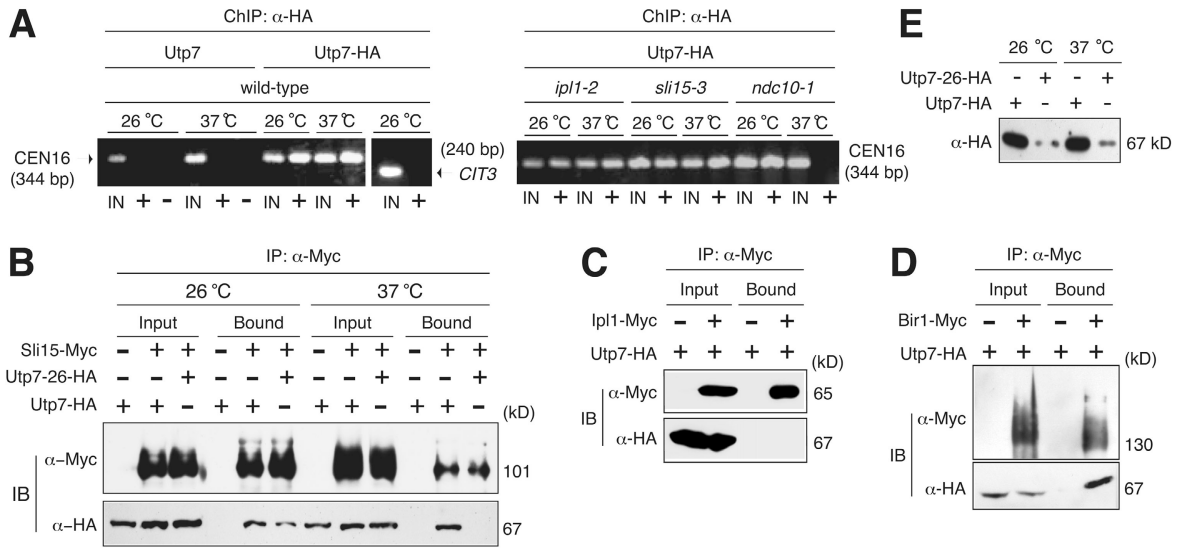

Figure 1. **Utp7 associates with Sli15, Bir1, and centromere DNA.** Cells were incubated at 26°C or, where indicated, shifted to 37°C for 3 h. (A) Chromatin immunoprecipitation (ChIP) was performed with anti-HA antibodies, using extracts from wild-type and mutant cells. CEN16 and *CIT3* sequences were amplified by PCR (28 cycles) from the total input chromatin (IN), antibody-immunoprecipitated samples (+), or mock-treated no-antibody controls (−). (B) Sli15-Myc was immunoprecipitated (IP) from extracts of wild-type cells that expressed Utp7-HA or from extracts of *utp7-26-HA* cells that also expressed Utp7-26-HA from a 2μ plasmid. Proteins were analyzed by immunoblotting (IB). (C and D) Similar to B, but Ipl1-Myc or Bir1-Myc was immunoprecipitated from wild-type cells that expressed Utp7-HA. (E) Extracts from cells expressing Utp7-HA or mutant Utp7-26-HA (from chromosomal locus) were immunoblotted with anti-HA antibodies.

is needed for the biogenesis of the 40S small ribosomal subunit (Dragon et al., 2002; Grandi et al., 2002). The nucleolus is also the site where the functions of an increasing number of proteins are regulated (Boisvert et al., 2007). The Cdc14 phosphatase is essential for mitotic exit. From G1 to metaphase, Cdc14 is sequestered and kept inactive in the nucleolus through its binding to Net1 as part of the RENT complex, which also contains the transcriptional repressor Sir2 (Straight et al., 1999; Visintin et al., 1999). During early anaphase, activation of the FEAR signaling network leads to the release of a fraction of the total pool of Cdc14 into the nucleoplasm (Stegmeier and Amon, 2004), where it dephosphorylates cyclin-dependent kinase (Cdk) substrates such as Sli15. At the end of M phase, activation of the mitotic exit network (MEN) leads to further release of Cdc14 into the nucleoplasm and cytoplasm, and Cdc14 dephosphorylates a key set of Cdk substrates to promote mitotic exit. The Cdc15 kinase and the Mob1–Dbf2 kinase complex of the MEN are also required for the redistribution and maintenance of the Sli15 complex on the anaphase spindle (Stoepel et al., 2005).

Here, we show that Utp7, a subunit of the SSU processome, functions not only in the nucleolus but also at kinetochores. Utp7 is required for proper chromosome segregation in addition to ribosome biogenesis. It associates with and regulates the localization of Sli15, Cdc14 and Net1. Interestingly, the regulation of Sli15 localization by Utp7 does not depend entirely on Cdc14 function.

Results

The *utp7-1* mutation is synthetic lethal with *ipl1-2*

We have previously performed a synthetic-lethal genetic screen for *sli* mutations (e.g., *sli15*) that cause *sli ipl1-2* double mutants to grow very slowly or become inviable at 26°C, the permissive growth temperature for *sli* and *ipl1-2* single mutants (Kim et al., 1999). One such *sli* mutation (*utp7-1*) resides within the essential *UTP7* gene, which encodes a WD40 repeat-containing component of the SSU processome that is essential for 35S pre-rRNA processing and 40S ribosomal subunit biogenesis (Dragon et al., 2002; Grandi et al., 2002). Utp7 is ∼40% identical over ∼480 amino acids to the human *BING4* gene product, which is present in preparations of nucleolus and mitotic spindle (Andersen et al., 2002; Sauer et al., 2005). The *utp7-1* mutant is partially defective in 40S ribosomal subunit biogenesis, whereas *ipl1-2* and *sli15-3* cells are not defective in this process even after a 3-h incubation at the restrictive growth temperature of 37°C (unpublished data). Furthermore, *ipl1-2* does not interact genetically with a number of mutations that compromise 40S (*rps27A*) or 60S (*rai1* or *spb2*) ribosomal subunit biogenesis. Thus, the synthetic-lethal relationship between *utp7-1* and *ipl1-2* is unlikely to be caused by a total failure in ribosome biogenesis in *utp7-1 ipl1-2* cells.

Utp7 associates with Sli15 and is present at kinetochores

In wild-type cells, Utp7 is present throughout the nucleus, but with especially high concentration in the nucleolus (Dragon et al., 2002; Grandi et al., 2002). In contrast, Ipl1 and Sli15 are not concentrated in the nucleolus (Biggins et al., 1999; Kim et al., 1999; He et al., 2001; Tanaka et al., 2002). To find out whether Utp7 might also function at kinetochores, we performed chromatin immunoprecipitation (ChIP) assays with cells that had Utp7 or one of four other SSU processome subunits (Nan1, Nop1, Rrp5, and Utp10) tagged by the HA epitope. Immunoprecipitation of Utp7-HA but not the other SSU processome subunits tested led to the coprecipitation of centromere (CEN) 16

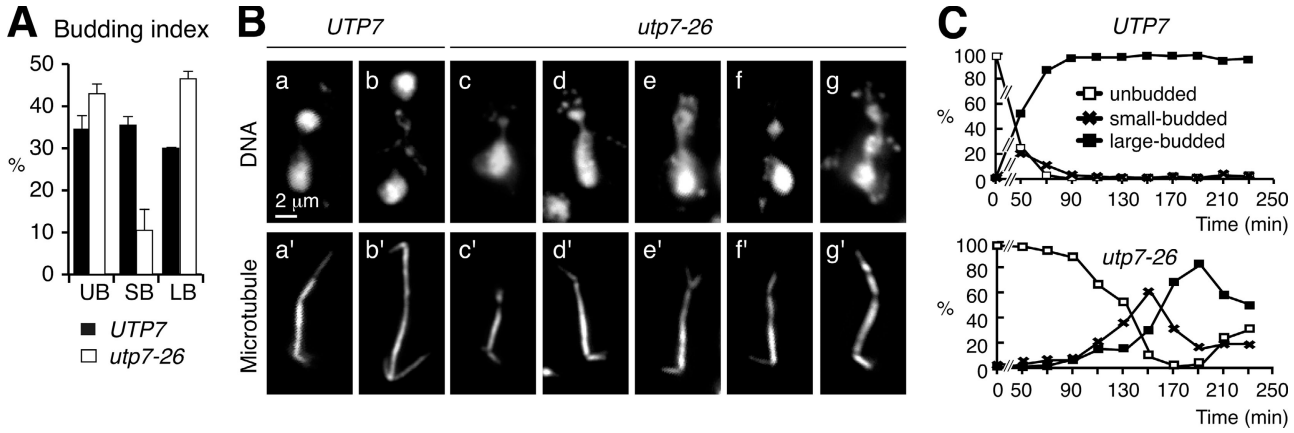

Figure 2. ***utp7-26* mutant cells missegregate chromosomes.** (A) Cells growing exponentially at 26°C were shifted to 37°C for 3 h. Budding index (UB, unbudded; SB, small-budded; LB, large-budded) of 100 cells each was scored. (B) Microtubule- and DNA-stained images of large-budded cells are shown. (C) Cells were arrested in G1 by α-factor at 26°C for 2 h, and then shifted to 37°C in the absence of α-factor but the presence of 15 μg/ml nocodazole. The budding index of 100 cells was scored at each time point.

(Fig. 1 A). The centromere association of Utp7-HA is specific because immunoprecipitation of Utp7-HA did not result in the coprecipitation of a *CIT3* sequence that is located ~1 kb from CEN16 (Fig. 1 A) or sequences from the more centromere-distal *IPL1* and *SLI15* loci (unpublished data). As for most other kinetochore proteins, the centromere association of Utp7-HA is abolished in *ndc10-1*^{ts} mutant cells that have a defective inner kinetochore (Fig. 1 A). Because we have not been able to detect cytologically the presence of Utp7 at kinetochores, our positive ChIP results suggested that Utp7 might associate transiently with kinetochores.

We also checked by immunoprecipitation assays whether Utp7 might associate with Sli15, Ipl1, and Bir1. Our results showed that Utp7-HA could be coprecipitated with Sli15-Myc and Bir1-Myc but not with Ipl1-Myc (Fig. 1, B–D). Purification of GST-Utp7 also led to the copurification of Sli15 and a very small amount of Ipl1 (that could be detected only after long exposure; unpublished data). Because Sli15 exists in at least two complexes in yeast, one containing Bir1 and the other containing Bir1 and Ipl1 (Cheeseman et al., 2002; Sandall et al., 2006; Widlund et al., 2006), our results suggested that Utp7 may associate preferentially with the Sli15 complex that does not contain Ipl1. However, we cannot rule out the possibility that the failure to precipitate Utp7-HA with Ipl1-Myc may be caused by technical problems, such as masking of the Myc-epitope on Ipl1 in the presence of Utp7-HA. In spite of the association of Utp7 with Sli15 and Bir1, the centromere association of Utp7-HA is not affected in *sli15-3* or *ipl1-2* mutant cells (Fig. 1 A). Because the centromere association of Ipl1 and mutant Sli15 is abolished in *sli15-3* cells (see Fig. 4 A; Emanuele et al., 2008), the kinetochore localization of Utp7 does not depend on the kinetochore localization of Sli15 or Ipl1.

utp7-26 mutant cells missegregate chromosomes

The *utp7-1* mutant that we identified is partially Cs$^-$ for growth at 13°C, but it does not exhibit any drastic cytological phenotypes. Thus, we generated additional *utp7* mutant alleles that confer a Ts$^-$ growth phenotype at 37°C. We have focused on the analysis of one such allele, *utp7-26*. At 26°C, *utp7-26* cells have a reduced growth rate and are partially defective in 40S ribosomal subunit biogenesis (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200802085/DC1). Similar to the *utp7-1* mutation, *utp7-26* is synthetic lethal with *ipl1-2* (and *sli15-3*) at 26°C.

To study chromosome segregation, we shifted asynchronous cultures of wild-type and *utp7-26* cells from 26 to 37°C. As controls, we also included the *enp1-1* and *krr1-17* SSU processome mutants (Sasaki et al., 2000; Chen et al., 2003). 3 h after shift, ~43% of *utp7-26* cells became unbudded, with the remainder becoming mostly large budded (Fig. 2 A). Because unbudded cells are known to accumulate upon depletion of Utp7 and other SSU components (Bernstein and Baserga, 2004), the slight enrichment in unbudded *utp7-26* cells might have resulted from the ribosome biogenesis defect of such cells (or it could have resulted from aneuploid cells generated by chromosome missegregation). Staining of microtubules and DNA revealed significant chromosome missegregation in *utp7-26* but not *enp1-1* or *krr1-17* cells (Fig. 2 B). Approximately 80% of the large-budded *utp7-26* cells were in anaphase and they showed heterogeneous cytological phenotypes, the most common and striking of which is uneven chromosome segregation (Fig. 2 B, panels c, d, and f). Approximately 68% of cells with separated chromosome masses had this phenotype. Chromosome missegregation was nonrandom, with the mother half of the cell receiving more chromosomal DNA ~80% of the time when uneven chromosome segregation occurred. This bias is opposite to that reported for *ipl1-2* and *sli15-3* cells (Tanaka et al., 2002). This unusual bias was not caused by abnormal segregation of the old spindle pole body to the mother half because the spindle pole body marker Spc42-RFP (Pereira et al., 2001) segregated properly (unpublished data).

In wild-type cells, a gap clearly existed between the separated chromosomal masses in cells that had an elongated spindle (Fig. 2 B, panels a and b). This gap was smaller or not present in many *utp7-26* cells, especially those with spindles that were

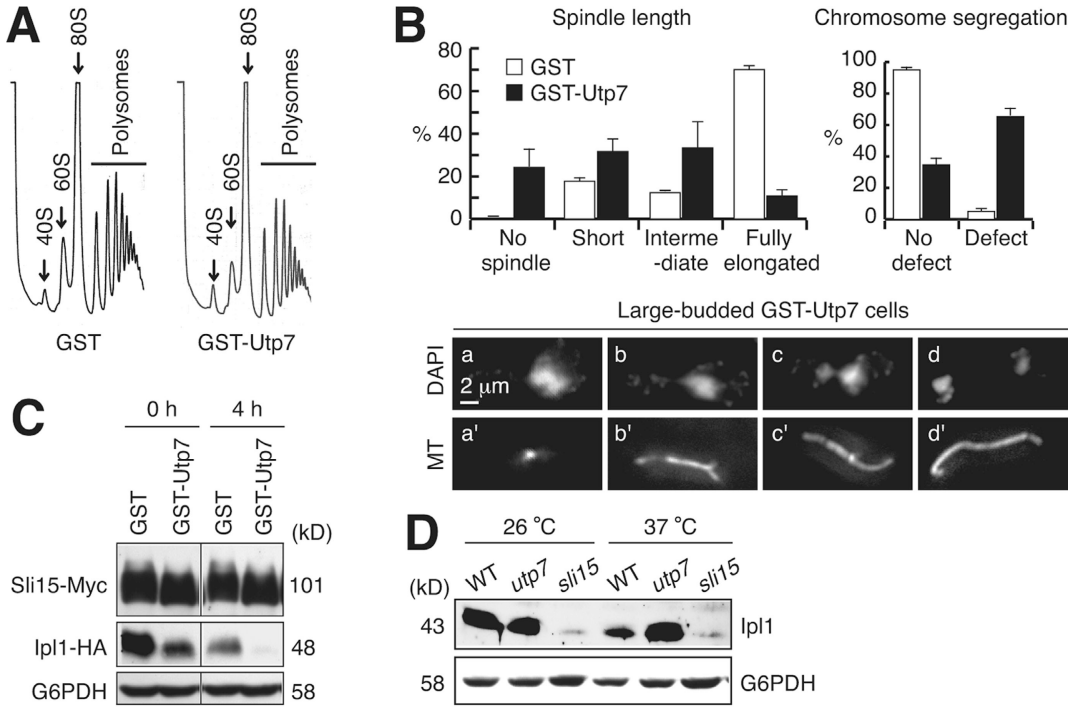

Figure 3. **Robust expression of GST-Utp7 leads to chromosome missegregation but not ribosome biogenesis defects.** (A) Expression of GST or GST-Utp7 was induced for 4 h at 30°C in exponentially growing wild-type cells that expressed Sli15-Myc, followed by ribosome profile analysis of extracts. (B) Cells in A were processed for DNA and microtubule staining. The spindle length of 100 large-budded cells and the chromosome segregation defect of 100 anaphase cells were scored. Short = typical metaphase spindle; fully elongated = typical telophase spindle. (C) Expression of GST or GST-Utp7 was induced in wild-type diploid cells heterozygous for *SLI15-MYC* and *IPL1-HA*. Extracts from these cells were immunoblotted with anti-Myc, anti-HA, or anti-G6PDH antibodies. (D) Extracts from cells that were incubated at 26°C or shifted to 37°C for 3 h were immunoblotted with anti-Ipl1 or anti-G6PDH antibodies.

not fully elongated (Fig. 2 B, panels d, e, and g). This result suggested that the segregation of some sister chromatids might have been delayed. This phenotype is not observed in *ipl1-2* or *sli15-3* mutant cells. Similar chromosome segregation defects were observed when *utp7-26* cells presynchronized in G1 by α-factor treatment at 26°C were allowed to enter the cell cycle at 37°C (unpublished data). Under such conditions, all *utp7-26* cells budded and progressed through the first cell cycle. Furthermore, when these G1-arrested cells were allowed to enter the cell cycle at 37°C in the presence of the microtubule-depolymerizing drug nocodazole, *utp7-26* cells, unlike wild-type cells, did not arrest as large-budded cells with unseparated chromosome mass (Fig. 2 C), thus suggesting that these cells are defective in mitotic spindle assembly checkpoint control.

Even at 26°C, *utp7-26* cells have greatly reduced amount of mutant Utp7-HA (Fig. 1 E), which likely is responsible for the partial defect in 40S ribosomal subunit biogenesis observed in these cells. Expression of mutant Utp7-HA from a 2μ plasmid restored mutant Utp7-HA level to roughly the endogenous wild-type level (Fig. 1 B) without suppressing the Ts^- phenotype or chromosome segregation defects of *utp7-26* cells (unpublished data). The association between Sli15-Myc and mutant Utp7-HA is temperature sensitive because mutant Utp7-HA was coprecipitated with Sli15-Myc from *utp7-26* cells that were incubated at 26°C, but not from cells that were incubated for 3 h at 37°C (Fig. 1 B). Thus, loss of association between Utp7 and Sli15 is correlated with chromosome missegregation.

Perturbation of Utp7 function can lead to chromosome missegregation without affecting ribosome profiles

Because the *utp7-26* mutant is defective in both ribosome biogenesis and chromosome segregation, it is possible that chromosome missegregation occurs in these cells as a consequence of the failure to synthesize certain proteins that are required for chromosome segregation. We consider this possibility unlikely because expression of GST-Utp7 from the *GAL1/10* promoter for 4 h had little effect on ribosome profiles in wild-type cells (Fig. 3 A) and yet caused heterogeneous chromosome segregation defects (Fig. 3 B). Approximately 24% of large-budded GST-Utp7–expressing cells had no spindle (Fig. 3 B, panel a'); and ∼65% of large-budded GST-Utp7–expressing cells that had an anaphase spindle segregated chromosomes unevenly to the two poles (Fig. 3 B, panels b–d). This latter phenotype is similar to that exhibited by many *utp7-26* cells.

Interestingly, expression of GST-Utp7 led to a dramatic reduction in the abundance of Ipl1-HA but not Sli15-Myc (Fig. 3 C). This reduction in Ipl1-HA abundance was unlikely to be caused by a defect in the synthesis of Ipl1-HA because the abundance of Ipl1 was also greatly reduced in *sli15-3* cells (which are not defective in ribosome biogenesis) and was not affected in *utp7-26* cells (Fig. 3 D). Metaphase spindles are unusually long in *ipl1* mutant cells (Biggins et al., 1999). In contrast, spindles fail to form in *ipl1 cin8* cells (Kotwaliwale et al., 2007). Because many GST-Utp7–expressing cells have no spindle, we suspect that GST-Utp7 expression affects not only the function

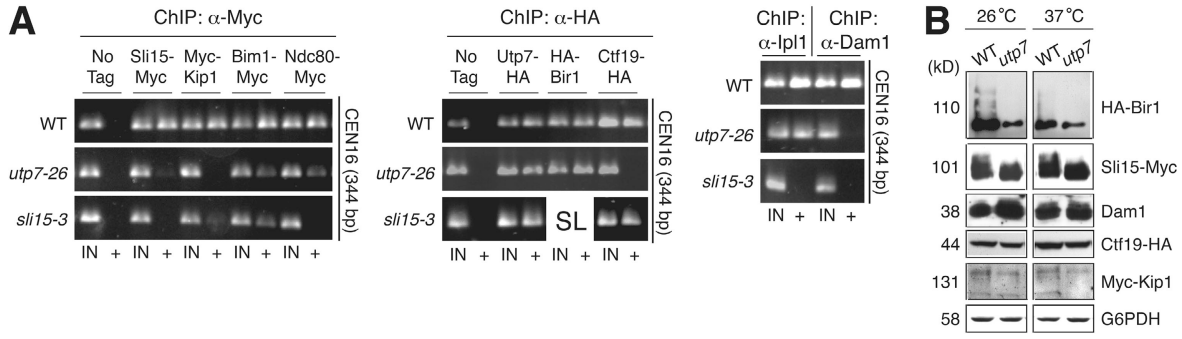

Figure 4. **Sli15 and other kinetochore proteins mislocalize in *utp7-26* cells.** (A) Chromatin immunoprecipitation (ChIP) was performed with the antibodies shown, using extracts from wild-type, *utp7-26*, or *sli15-3* cells that were incubated at 26°C and then shifted to 37°C for 3 h. PCR was performed as described in Fig. 1. (B) Immunoblotting of extracts from some of the cells used in A.

of Ipl1 (and Sli15) but also that of other proteins that regulate spindle microtubule stability or function. In any case, perturbation of Utp7 function by the robust expression of GST-Utp7 causes chromosome missegregation without affecting ribosome profiles (and likely protein synthesis).

Abnormal organization of Sli15 and multiple kinetochore proteins in *utp7-26* cells

To understand the molecular basis for chromosome missegregation in *utp7-26* cells, we used the ChIP assay to examine the centromere association of Utp7, Ipl1, Sli15, Bir1, and 11 additional kinetochore proteins (Bik1, Bim1, Dam1, Kip1, Slk19, and Stu2 of the outer kinetochore; Ctf19, Mtw1, and Ndc80 of the central kinetochore; and Cse4 and Ndc10 of the inner kinetochore) in wild-type and *utp7-26* cells. In these ChIP assays, we interpret a loss of CEN DNA PCR signal as a loss of centromere association for a specific immunoprecipitated protein, although other changes (e.g., masking of epitopes caused by changes in the organization of kinetochore proteins) might also cause a loss of PCR signal. Our results showed that after a 3-h incubation at 37°C, centromere association of Ipl1, HA-Bir1, Bik1-HA, Cse4-HA, Mtw1-Myc, Ndc10-HA, Slk19-HA, and Stu2-HA was unchanged in *utp7-26* cells (Fig. 4 A; unpublished data). Furthermore, in spite of the greatly reduced abundance of mutant Utp7-HA (Fig. 1 E), the centromere association of mutant Utp7-HA was also unchanged in *utp7-26* cells. In contrast, centromere association was abolished or greatly reduced for Sli15-Myc, Ctf19-HA, Dam1, and Myc-Kip1; and was significantly reduced for Bim1-Myc and Ndc80-Myc in *utp7-26* cells (Fig. 4 A). Thus, the organization of the outer and central kinetochore is greatly perturbed in *utp7-26* cells. In repeated experiments ($n \geq 2$), we have shown that the reduced centromere association of Sli15-Myc, Ctf19-HA, Dam1, and Myc-Kip1 in *utp7-26* cells was not caused by a significant reduction in the total cellular abundance of these proteins, and the abundance of HA-Bir1 was reduced without affecting its centromere association (Fig. 4 B).

Centromere association of Ipl1 and mutant Sli15-Myc was abolished in *sli15-3* cells (Fig. 4 A; Emanuele et al., 2008), thus indicating that kinetochore localization of Ipl1 requires functional Sli15 (i.e., similar to the situation with mammalian Aurora-B and INCENP; Klein et al., 2006). We were unable to check the centromere-association of Bir1 in *sli15-3* cells because *HA-BIR1* is synthetically lethal with *sli15-3*. However, centromere-association of Sli15-Myc but not Ipl1 and HA-Bir1 was greatly reduced in *utp7-26* cells (even when PCR was performed under more quantitative conditions; Fig. S2A, available at http://www.jcb.org/cgi/content/full/jcb.200802085/DC1). One possible interpretation for this result is that the greatly reduced amount of Sli15 that remains at kinetochores of *utp7-26* cells is sufficient to target Ipl1 and HA-Bir1 to kinetochores (e.g., with one molecule of Sli15 targeting more than one molecule of Ipl1 and Bir1). Alternatively, perturbations in the organization of the outer and central kinetochore described above somehow allow Ipl1 and HA-Bir1 to be targeted to kinetochores independent of Sli15.

Although the centromere association of Dam1, Myc-Kip1, Bim1-Myc, and Ndc80-Myc was similarly abolished or reduced in both *utp7-26* and *sli15-3* cells (Fig. 4 A; Emanuele et al., 2008), the centromere association of Ctf19-HA was abolished only in *utp7-26* cells, while the centromere association of Ipl1 was abolished only in *sli15-3* cells. Thus, the perturbed organization of kinetochore proteins observed in *utp7-26* cells is not caused entirely by the reduced centromere association of Sli15 in these cells. Utp7 likely regulates the localization of at least some kinetochore proteins by a Sli15-independent mechanism. This conclusion is consistent with differences in the chromosome missegregation phenotypes observed in *sli15-3* and *utp7-26* cells.

Altered microtubule localization of Sli15 in *utp7-26* cells

In preanaphase wild-type cells, Sli15 is concentrated at kinetochores, which tend to be located near the spindle poles. Sli15 abundance near the middle of the spindle is relatively low (Tanaka et al., 2002; Buvelot et al., 2003; Pereira and Schiebel, 2003). Upon anaphase onset, much of Sli15 redistributes to the length of the anaphase spindle. During no part of the cell cycle is Sli15 detected on cytoplasmic microtubules. Our immunostaining of Sli15-Myc in wild-type and *utp7-26* cells that had been incubated at 37°C for 3 h showed that Sli15-Myc was present abnormally on the cytoplasmic microtubules of *utp7-26* cells during all cell cycle stages (Fig. 5 A). Furthermore, *utp7-26* cells that had a short spindle and were presumably in preanaphase/

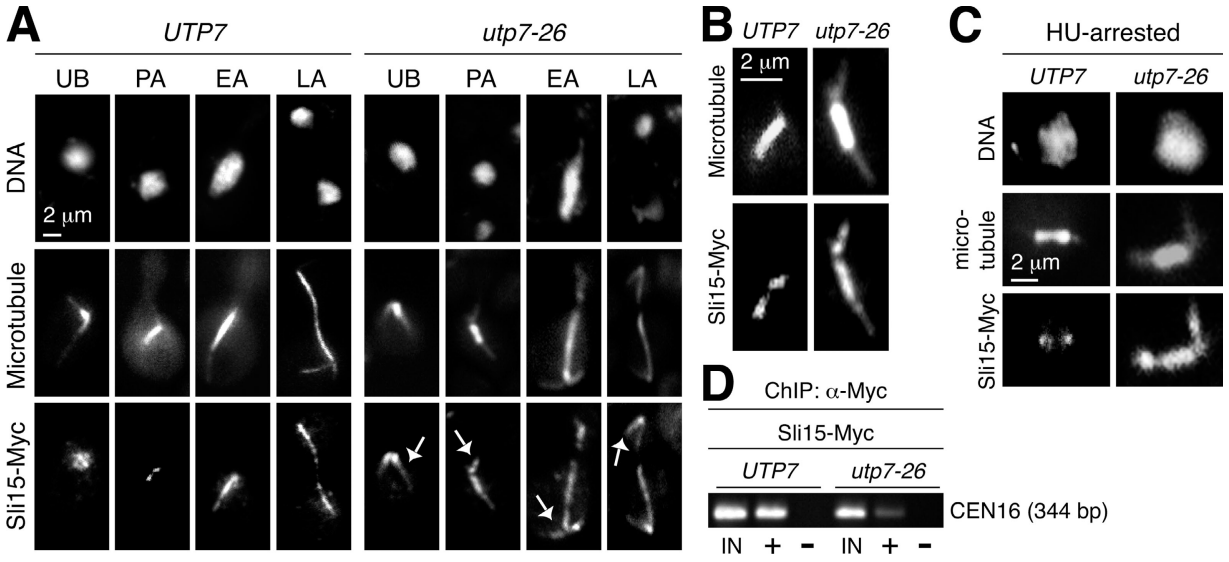

Figure 5. **Sli15 mislocalizes on microtubules in *utp7-26* cells.** (A) Cells expressing Sli15-Myc were incubated at 26°C and then shifted to 37°C for 3 h. Representative DNA-, microtubule-, and Sli15-Myc-stained images from unbudded (UB), preanaphase (PA), early anaphase (EA), and late-anaphase (LA) cells are shown. Arrows in some images mark Sli15-Myc signal on cytoplasmic microtubules. (B) Images of preanaphase cells from A are shown at higher magnification and with slight changes in contrast. (C) Similar to A, except cells were first incubated at 26°C for 1 h in the presence of HU and then shifted to 37°C for 2 h. (D) Chromatin immunoprecipitation (ChIP) was performed with anti-Myc antibodies, using extracts from cells shown in C. PCR was performed as described in Fig. 1.

metaphase had abnormally high levels of Sli15-Myc along the length of the spindle (Fig. 5 B).

To confirm that Sli15-Myc was concentrated abnormally on the spindle of preanaphase *utp7-26* cells, we treated cells at 26°C with hydroxyurea (HU) for 1 h to block cell cycle progression beyond S phase. These cells were then shifted to 37°C for 2 h in the presence of HU. Under such conditions, cells arrested uniformly as large-budded cells with a short preanaphase spindle. Sli15-Myc remained concentrated at kinetochores (near the spindle poles) in wild-type cells (Fig. 5 C). In contrast, Sli15-Myc was concentrated abnormally on the spindle and cytoplasmic microtubules of *utp7-26* cells. Furthermore, our ChIP assays also showed that the amount of Sli15-Myc that was associated with centromeres was reduced in *utp7-26* cells that were arrested by HU treatment (Fig. 5 D). Thus, Sli15-Myc is present abnormally on cytoplasmic microtubules during all cell cycle stages and is redistributed prematurely from kinetochores to the spindle in preanaphase *utp7-26* cells. Mutant Sli15 that associates prematurely with the spindle also targets Ipl1 to the spindle (Pereira and Schiebel, 2003). Thus, we also examined the localization of Ipl1-Myc in *utp7-26* cells. Unfortunately, the staining intensity for Ipl1-Myc was much weaker than that for Sli15-Myc even in wild-type cells. Nevertheless, it was clear that Ipl1-Myc, like Sli15-Myc, was present on cytoplasmic microtubules of *utp7-26* but not wild-type cells in all cell cycle stages (unpublished data). We were unable to determine definitively whether Ipl1-Myc was also concentrated prematurely on preanaphase spindles of *utp7-26* cells.

Mislocalization of Cdc14 and Net1 in *utp7-26* cells

From G1 to metaphase, the Cdc14 protein phosphatase is sequestered in the nucleolus through its binding to its inhibitor Net1. At the onset of anaphase, partial nucleolar release of Cdc14 is necessary for the dephosphorylation of Sli15 and the redistribution of the Sli15 complex from kinetochores to the spindle (Pereira and Schiebel, 2003). Because Sli15 is concentrated prematurely on the spindle of preanaphase *utp7-26* cells, we examined whether Cdc14 is no longer sequestered in the nucleolus of such cells. For this purpose, we performed the HU treatment and temperature-shift experiment described in Fig. 5 C with yeast cells that expressed the nucleolar protein Nop1-HA in combination with Cdc14-Myc or Net1-Myc. As expected, Cdc14-Myc and Net1-Myc were localized exclusively to the nucleolus in all HU-arrested preanaphase wild-type cells (Fig. 6 A, panels a' and d'). In contrast, Cdc14-Myc in HU-arrested preanaphase *utp7-26* cells was found throughout the entire nucleoplasm (~25%; Fig. 6 A, panels c and c') or in a substantial part of the nucleoplasm that contained much chromosomal DNA but no Nop1-HA (~75%; Fig. 6 A, panels b and b'). Thus, Cdc14-Myc is no longer localized exclusively in the nucleolus when Utp7 is inactivated at 37°C in HU-arrested preanaphase *utp7-26* cells. Furthermore, we have performed ChIP assays and have shown that Cdc14-Myc that was present in the nucleoplasm in HU-arrested preanaphase *utp7-26* cells associated with centromeres (Fig. 6 B).

Interestingly, the subcellular localization of Net1-Myc was also altered in most (~89%) HU-arrested preanaphase *utp7-26* cells (Fig. 6 A). The delocalization of Net1-Myc differed from that of Cdc14-Myc. Although Net1-Myc appeared to remain in the nucleolus in most (~91%) *utp7-26* cells, it was very often (~80%; Fig. 6 A, panels e and e') no longer organized into the rod-like structure typically seen in wild-type cells. In a small fraction (~9%) of *utp7-26* cells, a very low level of Net1-Myc (that was difficult to image and display) was also found throughout the nucleoplasm. However, Net1-Myc (unlike

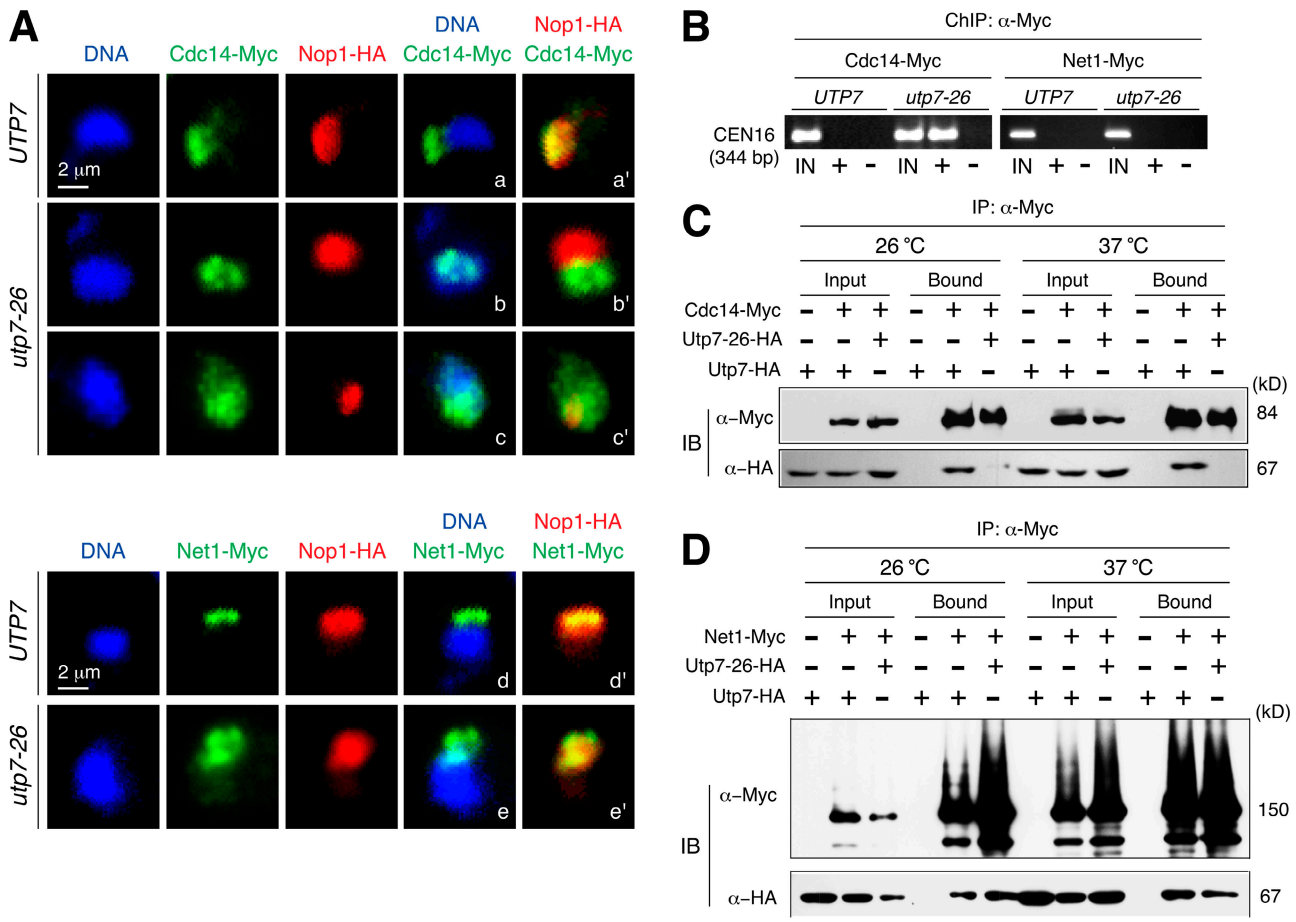

Figure 6. **Utp7 associates with and regulates the localization of Cdc14 and Net1.** (A) Cells expressing Nop1-HA in combination with Cdc14-Myc or Net1-Myc were first incubated at 26°C for 1 h in the presence of HU and then shifted to 37°C for 2 h. DNA was stained with DAPI; Nop1-HA with anti-HA antibodies; Cdc14-Myc and Net1-Myc with anti-Myc antibodies. (B) Chromatin immunoprecipitation (ChIP) was performed with anti-Myc antibodies, using extracts from cells shown in A. PCR was performed as described in Fig. 1. (C) Cdc14-Myc was immunoprecipitated (IP) from extracts of wild-type cells that expressed Utp7-HA or from extracts of *utp7-26-HA* cells that also expressed Utp7-26-HA from a 2μ plasmid. Cells were incubated at 26°C or shifted to 37°C for 3 h. Proteins were analyzed by immunoblotting (IB). (D) Similar to C, except that Net1-Myc was immunoprecipitated from cells that expressed Net1-Myc instead of Cdc14-Myc.

Cdc14-Myc) could not be detected by our ChIP assays at kinetochores of HU-arrested preanaphase *utp7-26* cells (Fig. 6 B).

In *net1* mutants, organization of the nucleolus is severely compromised and Nop1 localization is spread over the entire nucleoplasm (Straight et al., 1999; Shou et al., 2001). In contrast, Nop1-HA staining was still limited to the nucleolus in all HU-arrested preanaphase *utp7-26* cells (Fig. 6 A), although the shape of the Nop1-HA–stained area often appeared somewhat altered, perhaps reflecting the altered organization of Net1 in these cells. Thus, the *utp7-26* mutation alters localization of Cdc14-Myc and Net1-Myc without totally disrupting nucleolar structure.

We have also repeated these temperature-shift experiments with asynchronously growing cultures of wild-type and *utp7-26* cells. Our results showed that Cdc14-Myc was similarly delocalized in unbudded and small-budded *utp7-26* cells, and Net1-Myc was similarly delocalized through the entire cell cycle in *utp7-26* cells (unpublished data). Thus, Utp7 is required for the proper organization of Net1 and the nucleolar sequestration of Cdc14 from G1 to early anaphase. Because the abundance of Cdc14-Myc and Net1-Myc was not affected in *utp7-26* cells (unpublished data), the untimely nucleolar release of Cdc14 in these cells was not caused by an increased abundance of Cdc14 relative to its sequestering partner Net1.

We have also examined Sli15-Myc and Cdc14-Myc localization in the *enp1-1* and *krr1-17* SSU processome mutants (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200802085/DC1). After 3 h at 37°C, the localization of Sli15 was normal in all cell cycle stages and Cdc14-Myc remained sequestered in the nucleolus of preanaphase mutant cells (as in wild-type cells), thus indicating that the Sli15 microtubule localization and Cdc14 nucleolar sequestration defects observed in *utp7-26* were unlikely to be caused by a general defect in 40S ribosomal subunit biogenesis.

Physical interactions between Utp7, Cdc14, and Net1

Because both Cdc14 and Net1 are delocalized in *utp7-26* cells, we performed immunoprecipitation experiments to find out whether Utp7 might regulate the localization of Cdc14 and Net1 through an association with these proteins. Our results showed that precipitation of either Cdc14-Myc or Net1-Myc led to the

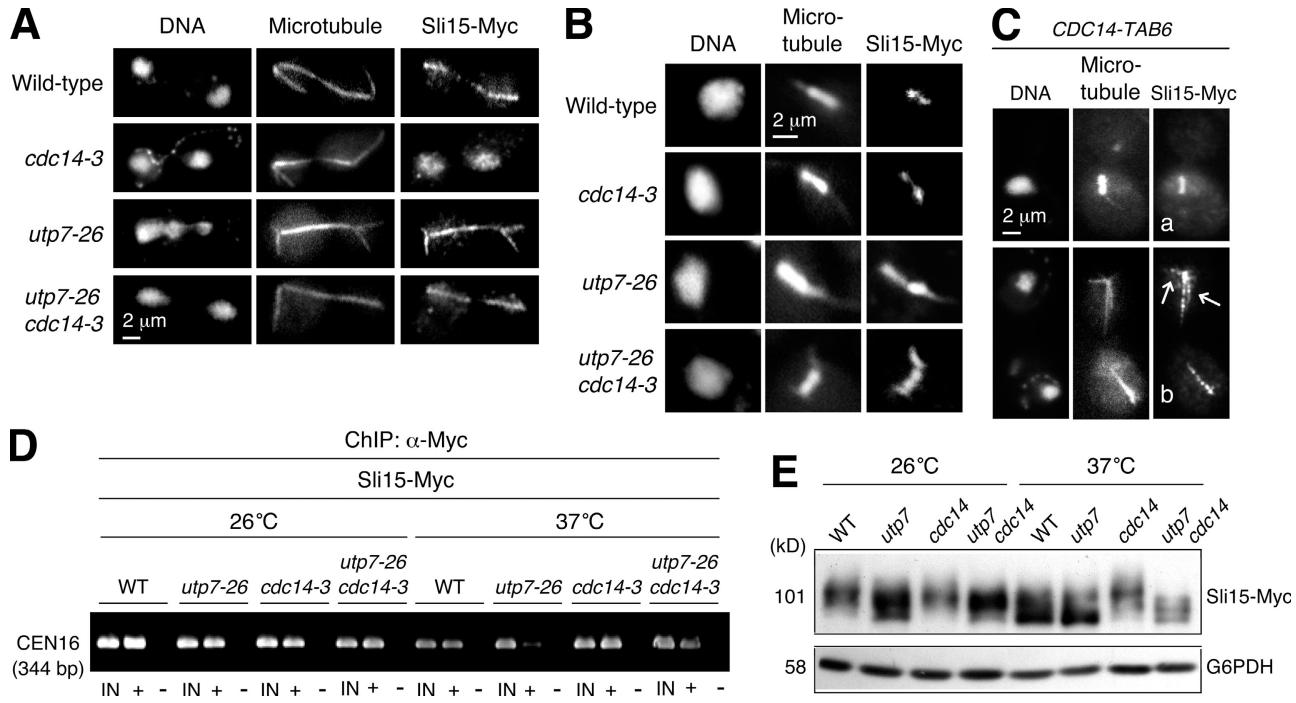

Figure 7. **Utp7 regulates Sli15 localization and phosphorylation by Cdc14-dependent and -independent mechanisms.** (A) Cells that expressed Sli15-Myc were incubated at 26°C and then shifted to 37°C for 3 h. Representative DNA-, microtubule-, and Sli15-Myc-stained images of cells in telophase are shown. (B) Similar to A, except cells were first incubated at 26°C for 1 h in the presence of HU and then shifted to 37°C for 2 h. (C) *CDC14-TAB6* cells that expressed Sli15-Myc were incubated at 26°C. Images of a large-budded preanaphase cell (top row) and a telophase cell (bottom row) are shown, with arrows marking Sli15-Myc signal on cytoplasmic microtubules. (D) Chromatin immunoprecipitation (ChIP) was performed with anti-Myc antibodies, using extracts from cells shown in B before and after the 2-h shift to 37°C. PCR was performed as described in Fig. 1. (E) Immunoblotting was performed with anti-Myc and anti-G6PDH antibodies, using extracts from cells shown in B before and after the 2-h shift to 37°C.

coprecipitation of Utp7-HA in wild-type cells (Fig. 6, C and D). Thus, Utp7 associates with Cdc14 and Net1. In parallel experiments, we also examined the ability of mutant Utp7-HA to associate with Cdc14-Myc or Net1-Myc in *utp7-26* cells that expressed mutant Utp7-HA from a 2μ plasmid. Our results showed that the association of mutant Utp7-HA with Net1-Myc was unaffected at 26°C or after 3 h at 37°C. In contrast, the association of mutant Utp7-HA with Cdc14-Myc was greatly reduced at 26°C and was undetectable after 3 h at 37°C. Thus, the failure to sequester Cdc14 in preanaphase *utp7-26* cells is correlated with the failure of mutant Utp7 to associate with Cdc14.

Cdc14 function is not essential for spindle localization of Sli15 in *utp7-26* cells

To find out whether the extra-nucleolar localization and thus activation of Cdc14 in preanaphase *utp7-26* cells is entirely responsible for the abnormal concentration of Sli15 on the spindle in such cells, we examined Sli15-Myc localization in wild-type, *utp7-26*, *cdc14-3^{ts}*, and *utp7-26 cdc14-3* cells. In our initial experiment, we shifted asynchronous cell cultures from 26 to 37°C for 3 h and focused on cells that were in telophase, the terminal arrest point for *cdc14-3* cells. As expected, Sli15-Myc was not concentrated on the spindle of *cdc14-3* cells but was found throughout the nucleoplasm (Fig. 7 A; Pereira and Schiebel, 2003). In contrast, Sli15-Myc was concentrated on the spindle of *utp7-26 cdc14-3* cells. A similar result was obtained with cells that were arrested in S phase by HU treatment before temperature shift (Fig. 7 B). Thus, Utp7 regulates the spindle localization of Sli15 in two redundant ways, one through its effect on the nucleolar sequestration of Cdc14 and another through a mechanism that is independent of Cdc14 function.

Sli15-Myc was present on cytoplasmic microtubules of *utp7-26* cells during all cell cycle stages (Fig. 5, A and B). In contrast, Sli15-Myc was present on cytoplasmic microtubules in HU-arrested preanaphase but not in telophase *utp7-26 cdc14-3* cells (Fig. 7, A and B). Thus, the cytoplasmic microtubule localization of Sli15-Myc is dependent on Cdc14 function in telophase but not in preanaphase *utp7-26* cells. Consistent with this observation, ectopic activation of Cdc14 in *CDC14-TAB6* cells (Shou and Deshaies, 2002; Bloom and Cross, 2007) resulted in the localization of Sli15-Myc on cytoplasmic microtubules in ~17% of telophase cells (with the remainder being hard to call due to very short cytoplasmic microtubules) (Fig. 7 C, b); whereas Sli15-Myc was undetectable on cytoplasmic microtubules in all large-budded preanaphase cells (Fig. 7 C, a). Thus, ectopic activation of Cdc14 by *utp7-26* or *CDC14-TAB6* causes Sli15-Myc to be localized on cytoplasmic microtubules during telophase, whereas a Cdc14-independent mechanism is responsible for the localization of Sli15-Myc on cytoplasmic microtubules in preanaphase *utp7-26* cells.

We have also performed ChIP assays to examine the association of Sli15-Myc with centromeres in HU-arrested preanaphase cells (to avoid potential cell cycle effects in different mutant cells). Mutational inactivation of Cdc14 increased centromere association of Sli15-Myc in *utp7-26* cells (Fig. 7 D). PCR performed under more quantitative conditions indicated

that centromere association of Sli15-Myc was not totally restored in *utp7-26 cdc14-3* cells (Fig. S2 B). Thus, the loss of Sli15-Myc from the preanaphase kinetochores in *utp7-26* cells is due partly, but not entirely, to the premature presence and functioning of Cdc14 at the kinetochores of these cells (Fig. 6 B).

Dephosphorylation of Sli15 by Cdc14 is required for the redistribution of Sli15 from kinetochores to the spindle (Pereira and Schiebel, 2003). In immunoblots of extracts prepared from asynchronous cell cultures, we noticed that Sli15-Myc was under-phosphorylated (and migrated faster in SDS-PAGE) in *utp7-26* cells (Fig. 4 B). Similarly, Sli15-Myc was also underphosphorylated in HU-arrested preanaphase *utp7-26* cells (Fig. 7 E). This was especially apparent in *utp7-26* cells that had not been shifted to 37°C. In contrast, Sli15-Myc was hyper-phosphorylated in preanaphase *cdc14-3* cells, especially after 2 h at 37°C. This was a surprising finding because the Cdc14 protein phosphatase is not thought to be active or present outside the nucleolus before anaphase onset and the *cdc14-3* mutation should thus have no effect on the phosphorylation state of its substrates at this cell cycle stage (see Discussion). Nevertheless, the phosphorylation state of Sli15-Myc in preanaphase *utp7-26 cdc14-3* cells was intermediate between that in wild-type and *cdc14-3* cells. Thus, Utp7 inactivation can lead to Sli15 dephosphorylation independent of Cdc14 function, and this dephosphorylation may be responsible for the concentration of Sli15 on the preanaphase (and anaphase) spindle of *utp7-26 cdc14-3* cells.

Partial suppression of *utp7-26* by *cdc14-3*

In addition to physical interactions, we also observed genetic interaction between Utp7 and Cdc14. The *cdc14-3* mutation suppressed the slow-growth phenotype of *utp7-26* cells at 26°C (Fig. S1 A). Because *utp7-26* cells are defective in 40S ribosomal subunit biogenesis at 26°C, we had assumed that the slow-growth phenotype of *utp7-26* cells at this temperature was caused by the ribosome biogenesis defect. We were surprised to learn that *utp7-26 cdc14-3* cells were similarly defective in 40S ribosomal subunit biogenesis (Fig. S1 B). Thus, the slow-growth phenotype of *utp7-26* cells at 26°C is probably caused by a defect that has nothing to do with ribosome biogenesis and *cdc14-3* suppresses this unknown defect of *utp7-26* cells. We suspect that a very small amount of Cdc14 might be released prematurely from the nucleolus in *utp7-26* cells even at 26°C. Because ectopic activation of Cdc14 before anaphase onset causes inappropriate dephosphorylation of Cdk substrates, including those involved in DNA replication (Bloom and Cross, 2007), reduction of Cdc14 function by the *cdc14-3* mutation would thus suppress the growth defect of *utp7-26* cells.

The spindles in telophase *cdc14-3* cells appeared relatively short and broken because spindle association of Sli15 is required for spindle stability during anaphase (Fig. 7 A; Pereira and Schiebel, 2003). In contrast, the spindles in telophase *utp7-26 cdc14-3* cells appeared intact and more normal in length, perhaps because the spindle association of Sli15-Myc was restored in these cells. In addition, the chromosome segregation defect (uneven chromosome segregation and lagging/bridging chromosomes) commonly seen in *utp7-26* cells was less commonly seen or severe in *utp7-26 cdc14-3* cells (Figs. 2 B and 7 A). For example, although ~68% of *utp7-26* cells with separated chromosome masses exhibited uneven chromosome segregation, only ~26% of *utp7-26 cdc4-3* cells with separated chromosome masses exhibited this phenotype. This observation suggests that the abnormal localization and functioning of Cdc14 in the nucleoplasm and at kinetochores of preanaphase *utp7-26* cells contributes to, but is not entirely responsible for, chromosome missegregation in these cells.

Discussion

We have shown here that the predominantly nucleolar ribosome biogenesis protein Utp7 associates with Sli15, Bir1, Cdc14, and Net1. It is present at kinetochores and is required for normal organization of kinetochore proteins and proper chromosome segregation. When Utp7 is inactivated, Cdc14 is no longer sequestered in the nucleolus before anaphase onset, and Sli15 becomes concentrated prematurely on preanaphase spindles and on cytoplasmic microtubules. However, premature targeting of Sli15 to the preanaphase spindle of *utp7-26* cells is not caused entirely by the premature nucleolar release and activation of Cdc14.

Utp7 and nucleolar sequestration of Cdc14

Cdc14 is sequestered and kept inactive from G1 to metaphase in the nucleolus as part of the RENT complex, which also contains Net1 and Sir2 (Straight et al., 1999; Visintin et al., 1999). We show here that inactivation of Utp7 in *utp7-26* cells leads to perturbation of the organization of Net1 mostly within the nucleolus and release of Cdc14 from the nucleolus through all cell cycle stages. Ribosome biogenesis is defective in *utp7-26* cells at 26 as well as 37°C; yet, dramatic failure in the nucleolar sequestration of Cdc14 occurs only at 37°C. Cdc14 nucleolar sequestration defect is apparent as soon as 10 min after shifting *utp7-26* cells to 37°C (unpublished data). The rapid onset of this phenotype is unlikely to be caused indirectly by protein synthesis problems because stopping ribosome biogenesis for 10 min would have only a minor effect on the total ribosome content in a cell. Furthermore, nucleolar sequestration of Cdc14 is not defective in the *enp1-1* and *krr1-17* SSU processome mutants.

Because Utp7 associates with Cdc14 and Net1, and these proteins are present at roughly similar abundance (~5,800, 8,550, and 1,590 molecules/cell for Utp7, Cdc14, and Net1, respectively; Ghaemmaghami et al., 2003), Utp7 potentially may function in a stable complex with Net1 and Cdc14, with its continual presence in this complex being essential for sequestering Cdc14 in the nucleolus. We consider this unlikely because the abundance of mutant Utp7 protein in *utp7-26* cells is very greatly reduced even at 26°C. Yet, dramatic failure of *utp7-26* cells to sequester Cdc14 occurs only at the restrictive growth temperature of 37°C. Thus, nucleolar sequestration of Cdc14 occurs even when the abundance of Utp7 is much lower than that of Net 1 and Cdc14. Furthermore, extensive mass spectrometric analysis of the purified RENT complex has not identified Utp7 as a stably bound component of this complex (Straight et al., 1999; Huang et al., 2006). Thus, we favor a model in

which Utp7 associates somewhat loosely or transiently with the RENT complex to regulate its stability. In *utp7-26* cells, association between Net1 and mutant Utp7 protein is unaffected; whereas the association between Cdc14 and mutant Utp7 is reduced at 26°C and becomes undetectable at 37°C. Thus, the ability of Utp7 to associate with Cdc14 appears to be important for the nucleolar sequestration of Cdc14 by Net1.

As part of the activation of the FEAR network upon anaphase onset (Stegmeier and Amon, 2004), the sister chromatid–separating protease separase associates with and down-regulates the activity of the $PP2A^{Cdc55}$ protein phosphatase toward Net1 (Queralt et al., 2006), thus allowing phosphorylation of Net1 by mitotic Cdk and partial nucleolar release of Cdc14 (Azzam et al., 2004). At the end of M phase, activation of the mitotic exit network (MEN) leads to further nucleolar release of Cdc14, probably involving phosphorylation of Cdc14 or Net1 by protein kinases in this network (Stegmeier and Amon, 2004). Utp7 potentially may inhibit untimely activation of the FEAR or MEN. However, we have not been able to detect changes in the electrophoretic mobility (and thus phosphorylation state) of Cdc14 and Net1 in *utp7-26* cells. Furthermore, unlike inactivation of Utp7, ectopic activation of the FEAR network (except by *net1* mutation) does not lead to nucleolar release of Cdc14 in G1 or S phase cells (Visintin et al., 2003; Stegmeier et al., 2004; Queralt et al., 2006). It remains to be determined whether untimely nucleolar release of Cdc14 in *utp7-26* cells requires functional components of the FEAR or MEN.

Utp7 and Sli15 localization

Inactivation of Utp7 in *utp7-26* cells affects the localization of Sli15 in two important ways. Sli15 becomes abnormally localized on cytoplasmic microtubules in all cell cycle stages. This abnormal localization potentially can be caused by a defect in the nuclear import or nuclear retention of a fraction of Sli15, which contains a putative nuclear localization signal and binds microtubules in vitro (Kang et al., 2001). Interestingly, the cytoplasmic microtubule localization of Sli15 is dependent on Cdc14 function in telophase but not preanaphase *utp7-26* cells. It remains to be determined how Utp7 and Cdc14 prevent cytoplasmic microtubule localization of Sli15.

Sli15 becomes concentrated on the spindle prematurely before anaphase onset in *utp7-26* cells. This is not a surprising result because Cdc14 is not sequestered in the nucleolus of these *utp7-26* cells, and ectopic Cdc14 activation and dephosphorylation of Sli15 is known to be sufficient to redistribute Sli15 from kinetochores to the spindle (Pereira and Schiebel, 2003). However, Cdc14 function is not absolutely required for the concentration of Sli15 on the spindle of HU-arrested preanaphase or late-anaphase *utp7-26* cells. Thus, Utp7 regulates the spindle localization of Sli15 in two redundant ways, one through its effect on the nucleolar sequestration of Cdc14 and another through a mechanism that is independent of Cdc14 function.

We have shown that Sli15 is hyper-phosphorylated in HU-arrested preanaphase *cdc14-3* cells. This surprising result suggests that a small amount of active Cdc14 may actually be present outside the nucleolus before anaphase onset, or that Sli15 somehow has access to active nucleolar Cdc14 in wild-type cells.

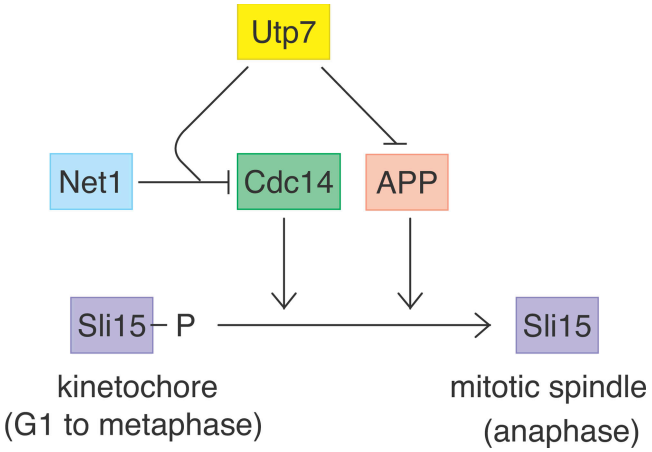

Figure 8. **Model for the regulation of Sli15 phosphorylation and localization by Utp7.** APP = another protein phosphatase. In an alternative model, Utp7 positively regulates an unknown kinase that phosphorylates Sli15.

Although Sli15 is undetectable in the nucleolus of yeast cells, the human homologue of Sli15 (INCENP) is present in the nucleolus of interphase cells (Rodriguez et al., 2006; Wong et al., 2007) and in highly purified preparations of nucleoli (Andersen et al., 2005). Thus, Sli15 (Ipl1 and Bir1) may also shuttle transiently through the yeast nucleolus in preanaphase cells.

Importantly, the phosphorylation state of Sli15 in HU-arrested preanaphase *utp7-26 cdc14-3* cells is intermediate between that of wild-type and *cdc14-3* cells. Thus, Utp7 can regulate Sli15 phosphorylation independent of Cdc14 function, suggesting that Utp7 also negatively regulates another protein phosphatase (APP) that dephosphorylates Sli15 (Fig. 8). In this model, APP becomes hyperactive, or active at an inappropriate location or cell cycle stage (e.g., before anaphase onset) in *utp7-26* cells. When de-regulated, APP dephosphorylates Sli15 at (some of) the Cdk sites normally dephosphorylated by Cdc14, thus leading to the association of Sli15 with the spindle even when Cdc14 is inactive (e.g., in preanaphase *utp7-26* cells or anaphase *utp7-26 cdc14-3* cells). Interestingly, Glc7, the catalytic subunit of protein phosphatase 1, is concentrated in the nucleolus where Utp7 is concentrated (Bloecher and Tatchell, 2000), and we have shown previously that Glc7 counteracts Ipl1 function (Francisco et al., 1994; Tung et al., 1995). Thus, Glc7 is a potential regulatory target of Utp7. In an alternative model, Utp7 may positively regulate an unknown kinase that phosphorylates Sli15.

Utp7 and chromosome segregation

The centromere association of at least six central or outer kinetochore proteins (Bim1, Ctf19, Dam1, Kip1, Ndc80, and Sli15) is greatly reduced or totally abolished in *utp7-26* cells. This severe perturbation of kinetochore structure almost certainly contributes to chromosome missegregation in *utp7-26* cells. The premature nucleolar release of Cdc14, which is known to cause Sli15 to redistribute from kinetochores to the spindle (Pereira and Schiebel, 2003), potentially explains the reduced centromere association for Sli15 in *utp7-26* cells. Because the centromere association of Bim1, Dam1, Kip1, Ndc80, and Sli15 is abolished

or reduced in *sli15-3* cells (Emanuele et al., 2008), the reduced centromere association of Sli15 in *utp7-26* cells would in turn lead to the reduced centromere association of Bim1, Dam1, Kip1, and Ndc80 in *utp7-26* cells. However, the centromere association of Ctf19 is totally abolished in *utp7-26* but is unaffected in *sli15-3* cells, and the centromere association of Ipl1 is totally abolished in *sli15-3* but is unaffected in *utp7-26* cells. Thus, the effect of the *utp7-26* mutation on kinetochore organization does not act entirely through Sli15. It is possible that the premature nucleolar release and centromere association of Cdc14 in *utp7-26* cells directly contributes to the loss of centromere association for Ctf19 (and possibly other kinetochore proteins). Premature nucleolar release of Cdc14 may also contribute to chromosome missegregation by affecting microtubule dynamics through its action on spindle-associated proteins such as Fin1 (Higuchi and Uhlmann, 2005; Woodbury and Morgan, 2007).

For four reasons, we think that the chromosome segregation defects observed in *utp7-26* cells are unlikely to be caused entirely by the untimely activation of Cdc14 in these cells. First, inactivation of Cdc14 by the *cdc14-3* mutation only partially suppresses the chromosome segregation defects of *utp7-26* cells. Second, inactivation of Cdc14 by the *cdc14-3* mutation only partially restores the centromere association of Sli15 in *utp7-26* cells. Third, ectopic activation of Cdc14 by the *CDC14-TAB6* mutation does not lead noticeably to chromosome missegregation (unpublished data). Fourth, Utp7 itself associates with kinetochores. Thus, Utp7 most likely also regulates chromosome segregation through a mechanism that is independent of untimely Cdc14 activation. Because Utp7 associates with Sli15 and Bir1, and expression of GST-Utp7 reduces abundance of Ipl1, Utp7 may compete with Ipl1 for binding to Sli15 and thus directly regulates Sli15 complex function at kinetochores. In addition, overexpression of Glc7 causes severe chromosome missegregation (Francisco et al., 1994; Tung et al., 1995). If Utp7 negatively regulates Glc7 (Fig. 8), de-regulated Glc7 in *utp7-26* cells also would contribute to chromosome missegregation in these cells.

Ribosome biogenesis and other cellular processes

Ribosome biogenesis is an extremely energy-intensive process, consuming up to 80% of the energy of a rapidly growing eukaryotic cell. Consequently, ribosome biogenesis must be regulated according to all the other needs of a cell (Warner, 1999). Interestingly, many ribosome biogenesis genes (including *UTP7*) are up-regulated in aneuploid yeast cells (Torres et al., 2007). In this context, the physical and functional interactions between Utp7, the Sli15/INCENP and RENT complexes suggest a potential functional link of ribosome biogenesis to the processes of chromosome segregation and cell cycle control. Because Utp7, Sli15, and Cdc14 are conserved from yeast to humans, this potential functional link may extend to other organisms. Cbf5, another conserved and predominantly nucleolar ribosome biogenesis protein (Cadwell et al., 1997; Grandi et al., 2002), binds yeast centromere DNA in vitro and interacts genetically with the kinetochore protein Ndc10 (Jiang et al., 1993). Thus, additional ribosome biogenesis proteins may also be involved in chromosome segregation.

Materials and methods

Strains, plasmids, media, and genetic techniques

The yeast strains and plasmids used in this study are listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200802085/DC1). Yeast genetic manipulation and the preparation of yeast growth media were performed as described previously (Burke et al., 2000). Expression of GST or GST-Utp7 under the *GAL1/10* promoter control was performed essentially as described by Kim et al. (1999). For ribosome profile analysis, exponentially growing cells at OD_{600} = 0.3 were treated with 0.2 mg/ml cycloheximide for 1 min. Extracts prepared from these cells were sedimented through a sucrose gradient (7–47%), and the ribosome profiles were determined by absorbance of gradient fractions at 260 nm.

Molecular cloning of *UTP7*

We have previously used a colony-sectoring assay to identify *sli* mutations that confer a lethal or very slow growth phenotype only when combined with the *ipl1-2* mutation (Kim et al., 1999). The *sli12-1* mutation confers a cold-sensitive (Cs^-) growth phenotype at 13°C. The *SLI12* gene was cloned by complementation of the Cs^-, nonsectoring, and 5-FOA–sensitive phenotypes of *ade2 ade3 ura3 leu2 ipl1-2 sli12-1* cells that contained an *IPL1-URA3-ADE3*-plasmid (CCY715-19D), using a yeast genomic library constructed in the *LEU2*-CEN-plasmid p366 (provided by Phil Hieter, University of British Columbia, British Columbia, Canada). Plasmid subcloning and genetic linkage analysis showed that the *sli12-1* mutation resides in the *UTP7* gene. Thus, we renamed the *sli12-1* mutation *utp7-1*.

Construction of the temperature-sensitive *utp7-26* mutant allele

T3 and T7 primers (Promega) were used in a standard 30-cycle PCR reaction with *Taq* DNA polymerase (Promega) to amplify the *UTP7* gene present on the *LEU2*-CEN-plasmid pCC863. Approximately 0.5 µg of the ~3.6-kb PCR product and ~0.1 µg of the ~7.5-kb BglII–XbaI fragment of unmutagenized pCC863 were used to transform the yeast strain CCY1398-11B, which contained *UTP7* on a *URA3*-CEN-plasmid (pCC1000) as the only source of *UTP7*. Leu^+ Ura^+ transformants were selected at 26°C on supplemented SD medium. Transformants were tested for their ability to grow at 26 and 37°C on supplemented SD medium lacking leucine but containing uracil and 5-FOA (1 g/L for 26°C and 0.5 g/L for 37°C). Transformant colonies that could grow on 5-FOA plates at 26 but not at 37°C were chosen. After colony purification by streaking, such Leu^+ Ura^+ transformants were retested for their differential sensitivity to 5-FOA at 37 but not at 26°C. The *LEU2*-plasmids were recovered from 5-FOA–sensitive transformants into *Escherichia coli*. The ability of each plasmid to support growth of CCY1398-12B (which contained *UTP7* on a *URA3*-CEN plasmid [pCC1000] as the only source of *UTP7*) on 5-FOA plates at 26 and 37°C was retested. Cells from 26°C FOA plates were then tested for temperature sensitivity at 37°C on YEPD. From ~60,000 Leu^+ Ura^+ transformants screened, 25 plasmids containing temperature-sensitive *utp7* alleles (*utp7-10* to *utp7-34*) were isolated.

Preliminary cytological studies of these *utp7* mutants that had plasmid-borne *utp7* allele indicated two major phenotypes after 3 h at 37°C: enrichment of unbudded cells or large-budded cells with a short spindle that was suggestive of mitotic spindle assembly checkpoint activation, with different mutants exhibiting one or both of these phenotypes. To integrate each *utp7* mutant allele on the chromosome, we cloned the ~3.5-kb SacI–BamHI fragment that contains each mutant allele into the SacI–BamHI sites of the integrating *LEU2*-plasmid pRS305. The resulting plasmid was linearized at the unique SphI site and used to transform CCY1398-12B, resulting in the integration of the *utp7* mutant allele downstream of the *utp7-Δ* locus on the chromosome. To our surprise, mutants containing a single copy of chromosomally integrated *utp7* as the only source of *UTP7* either had no observable cytological phenotype or were arrested mostly as unbudded cells after 3 h at 37°C. We reasoned that the difference in phenotype between cells with CEN-plasmid–borne and chromosomally integrated *utp7* mutant alleles might have to do with differences in the copy number of the *utp7* mutant allele. Thus, we cloned eight of the *utp7* alleles into the XbaI–BamHI sites of the *HIS3*-integrating plasmid pRS303. The resulting plasmid was linearized by BssHII and used to integrate a second copy of *utp7* onto the chromosome. One such doubly integrated mutant (*utp7-26*) exhibited a strong chromosome missegregation phenotype and is the subject of this study.

Protein and immunological techniques

Immunoprecipitation, immunoblotting, and chromatin immunoprecipitation were conducted as described previously (Kang et al., 2001). The antibodies

used were: rabbit anti-G6PDH (Sigma-Aldrich), mouse monoclonal 12CA5 anti-HA (Covance), mouse monoclonal 9E10 anti-Myc (Covance), guinea pig anti-Ipl1 (Kim et al., 1999), and guinea pig anti-Dam1 (Cheeseman et al., 2002).

Cytological techniques

Microscopy was performed using an Axioscope (Carl Zeiss, Inc.) with a 100X phase objective (NA = 1.3). Images were captured using a MicroMax CCD camera (Princeton Instruments) and processed using IPLab spectrum (BD Biosciences). Immunofluorescent staining of yeast cells was performed as described previously (Pringle et al., 1989), using FITC- or rhodamine-conjugated anti–rat or anti–mouse secondary antibodies (MP Biomedicals/Cappel). For cell synchrony experiments, α-factor (custom synthesized) was used at 10 μg/ml and hydroxyurea (Sigma-Aldrich) was used at 0.2 M.

Online supplemental material

Table S1: plasmids and yeast strains used in this study. Fig. S1: *cdc14-3* suppresses the slow growth but not ribosome biogenesis defect of *utp7-26* cells at 26°C. Fig. S2: centromere association of Sli15 but not Ipl1 or Bir1 is reduced in *utp7-26* cells, and this centromere association is partially restored in *utp7-26 cdc14-3* cells. Fig. S3: Sli15 and Cdc14 localize properly in *enp1-1* and *krr1-17* cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200802085/DC1.

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