Cdc15 Phosphorylates the C-terminal Domain of RNA Polymerase II for Transcription during Mitosis*

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In eukaryotes, the basal transcription in interphase is orchestrated through the regulation by kinases (Kin28, Bur1, and Ctk1) and phosphatases (Ssu72, Rtr1, and Fcp1), which act through the post-translational modification of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. The CTD comprises the repeated Tyr-Ser-Pro-Thr-Ser-Pro-Ser motif with potential epigenetic modification sites. Despite the observation of transcription and periodic expression of genes during mitosis with entailing CTD phosphorylation and dephosphorylation, the associated CTD specific kinase(s) and its role in transcription remains unknown. Here we have identified Cdc15 as a potential kinase phosphorylating Ser-2 and Ser-5 of CTD for transcription during mitosis in the budding yeast. The phosphorylation of CTD by Cdc15 is independent of any prior Ser phosphorylation(s). The inactivation of Cdc15 causes reduction of global CTD phosphorylation during mitosis and affects the expression of genes whose transcript levels peak during mitosis. Cdc15 also influences the complete transcription of clb2 gene and phosphorylates Ser-5 at the promoter and Ser-2 toward the 3’ end of the gene. The observation that Cdc15 could phosphorylate Ser-5, as well as Ser-2, during transcription in mitosis is in contrast to the phosphorylation marks put by the kinases in interphase (G1, S, and G2), where Cdk7/Kin28 phosphorylates Ser-5 at promoter and Bur1/Ctk1 phosphorylates Ser-2 at the 3’ end of the genes.

The large subunit of eukaryotic RNA polymerase II (RNAPII) contains an unstructured tail of heptad repeats (YSPTSPS) termed the CTD, the copy number of which generally increases with the complexity of the organism. The dynamic post-translational modifications and structural plasticity enable the CTD to serve as a binding platform for a variety of regulatory factors required for mRNA biogenesis (1, 2). For the basal transcription in interphase, the Kin28/Cdk7 subunit of TFIIH complex phosphorylates CTD at Ser-5 (also Ser-7) and helps in the preinitiation complex formation, transcription initiation, and efficient mRNA capping. During early elongation, Ser-2 kinases (Cdk9/Bur1) act on the prephosphorylated CTD and help the subsequent progression of transcription and mRNA biogenesis (3–5). The phosphatases Ssu72, Rtr1, and Fcp1 remove phosphorylated Ser-5/Ser-7, Ser-5, and Ser-2, respectively, of CTD during interphase (1, 2, 6).

The CTD phosphorylation and its role during mitosis remains obscure. A large fraction of RNAPII is no longer associated with chromatin after the onset of mitosis however, a small fraction is recruited to the selective genes and remains transcriptionally active. The genes carrying a paused or elongating RNAPII continue and finish transcription before the anaphase (7, 8). The temporary cessation of transcription is mostly relieved and transcription resumes from the telophase (9, 10). Transcription in mitosis was considered arrested mainly because of the presence of highly condensed chromatin, dissociation of the key components of transcription complex, and inactivation of the kinase subunit (CAK) of TFIIH (11–14). Conversely, the presence of decondensed chromatin fibrils, chromatin remodelers, RNAPII-associated transcription factors on centromere satellite transcripts and intergenic spacer sequences, identification of Cdc14 as a mitotic CTD phosphatase, and the phosphorylated Ser-2 and Ser-5 of CTD support the presence of transcriptionally engaged RNAPII for transcription during mitosis (7, 15–18). The genome-wide transcriptome analysis in yeast has also provided clues of widespread periodic expression of genes during G2/M and mitosis (19, 20).

In Saccharomyces cerevisiae, Cdc28 regulates cell cycle by phosphorylating a large number of substrates. During mitosis, the reversal of Cdc28-dependent phosphorylation of client proteins is regulated by Cdc14 and thus a faithful mitotic exit (21, 22). Cdc28 kinase activity also boosts transcription of a large subset of genes in interphase by directly stimulating the basal transcription machinery where in association with Kin28 (Kin28 primes the phosphorylation by Cdc28), it influences the Ser-5 phosphorylation of the CTD (23). Cdc15 is a key coordinator of multiple signals, including the activation of Cdc14 and is essential for the accurate exit from mitosis (24, 25). Cdc15 is present in fungi and basal eukaryotes but absent in human. It is
a subfamily of STE11 kinase, and its role in the mitotic exit network appears to be replaced by Mst2 protein in humans (26).

Here we are reporting the Cdc15 of budding yeast, a potential kinase phosphorylating CTD of RNAPII and its role in transcription during mitosis.

**Results**

**Cdc15 Phosphorylates Ser-2 and Ser-5 of CTD**—The comparative analysis of the genes transcribed during interphase suggests the inevitable role of TFIIH. The blocking of Kin28 from phosphorylating the CTD effectively blocks promoter release and disables transcription initiation by RNAPII (27). The CAK complex (Kin28-Ccl1-Tfb3) of TFIIH is inactive at non-permissive temperatures, as well as during mitosis (11, 28), and hence transcription is not possible without Ser-5 phosphorylation by other kinase(s).

The probable yeast kinases were purified from the *S. cerevisiae* using standard TAP affinity method, and its ability to phosphorylate a recombinant CTD was examined (29). Apart from the known CTD kinases (Kin28, Ctk1, and Bur1), only Cdc15 efficiently phosphorylated Ser-2 (Ser(P)-2) and Ser-5 (Ser(P)-5) of CTD (Fig. 1A). The other kinases that play an important role during mitosis (Dbf2, Dbf20, Cdc5, and Cdc28) could not phosphorylate CTD to a detectable limit. The entire list of kinases purified and tested for CTD kinase activity is provided in Table 1. To confirm the specificity of Cdc15 toward CTD, we examined its ability to phosphorylate the mutant CTD, wherein a single point mutation for Ser-2, Ser-5, or Ser-7 was carried out (Fig. 1B). The results show that an alanine substitution of CTD at Ser-2 (S2A or A2) or Ser-5 (S5A or A5) affects the specific Cdc15 activity toward CTD, whereas Ser-7 mutation (S7A or A7) does not affect the phosphorylation of Ser-2 or Ser-5 resi-
Cdc15 Is a Mitotic CTD Kinase

TABLE 1

The purification and screening of kinases for the CTD phosphorylations

| Kinase | Purification | Ser(P)-2 | Ser(P)-5 |
|--------|--------------|----------|----------|
| Kin28  | +++          | –        | ++       |
| Bur1   | +++          | +        | ++       |
| Cdk1   | +++          | +        | ++       |
| Cdc15  | +++          | +        | ++       |

Cdc15 Temperature-sensitive Mutant Strain Has Reduced Ser(P)-5 and Ser(P)-2 Levels in Vivo during Mitosis—Although the analog-sensitive kinase allows for rapid, reversible, and specific inhibition of individual kinases in cells, many times it also encountered reduced activity, transient action, nonspecific inhibition, and lack of cellular lethality (30, 31). Because the cell cycle regulation has extensively been studied by temperature-sensitive mutants, we also studied the change in the global CTD phosphorylation using temperature-sensitive Cdc15 mutant (Cdc15-2). At non-permissive temperature, the Cdc15 mutant is arrested at telophase because of the affected mitotic exit network. Cdc15-2 has been used as a control to see the effect of Cdc14 (mitotic CTD phosphatase) in the expression of intergenic spacer sequence and Y element of the right arm of chromosome IV (TEL4R-Y) during mitosis (16). Here we analyzed the change in the global Ser-5 and Ser-2 phosphorylation of CTD in the cell lysates of Cdc15-2 strain at the permissive and non-permissive temperatures (Fig. 2). In case of non-functional Cdc15, a significantly reduced level of Ser(P)-5 and Ser(P)-2 was observed as compared with the wild type cells during mitosis (Fig. 2, A and B, Arrest). The comparative reductions in the Ser(P)-5 levels between the arrested and released cells for Cdc15 mutants were highly significant. The decrease in the CTD phosphorylation in the arrested cells could be due to the inactive Cdc15. At similar experimental conditions, there was also a significant reduction in Ser-2 phosphorylation.

To confirm that the above results are specific to the Cdc15 inactivation, we also examined changes in the global CTD phosphorylation in case of mutant mitotic CTD phosphatase, Cdc14 (temperature-sensitive Cdc14 or Cdc14-1). Comparison of the arrested cells of the wild type and Cdc14-1 strains showed no significant changes in the CTD phosphorylation (Fig. 2D and E, Arrest). To examine whether the cells can recover from the arrest and whether Cdc15 could phosphorylate the CTD again at the permissive temperature, we checked the comparative CTD phosphorylation in the released cells and observed that the cells regained their CTD phosphorylation state in all the cases (Fig. 2, A, B, D, and E, Release). The insignificant change in the Ser(P)-5 level for Cdc14-1 at the non-permissive and permissive temperatures signifies the continuation of CTD phosphorylation and dephosphorylation cycle essential for transcription. Here the levels of Rpb3 remain insignificantly changed in all the cases (Fig. 2, C and F). The quantification of Western blot signals also affirms the significant reduction in the phosphorylation level in case of arrested Cdc15-1 cells (Fig. 2, A and B, upper panels), whereas in other cases it remains unchanged (Fig. 2, C–F, upper panels).

The effect of permissive and non-permissive temperature on the mutant strain growth shows a similar lack of growth at 37 °C (Fig. 3A). The Cdc15-2 and Cdc14-1 have a common origin, arrest cell by the same mode at telophase, and their morphologies show characteristic dumbbell feature (Fig. 3B). They mostly occupy similar locus and have together been studied extensively for the gene expression analysis (16).

Transcript Level Decreases in the Cdc15 Temperature-sensitive Cells—The genome-wide transcriptome analysis in yeast has provided clues of widespread periodic expression of genes. During mitosis, the expression of many genes periodically changes and accomplishes before anaphase; however, the gene regulatory players remain obscure (19). Various studies suggest the presence of certain transcription factors associated with Rnapii during mitosis that act as bookmarks for subsequent gene activation (32). In addition, various genes whose transcription begin in interphase have their expression level peak during mitosis. In such cases, it is assumed that the CTD phosphorylated (especially Ser-2) during interphase is sufficient to
facilitate the remaining transcription during mitosis (19, 20). The genes whose expression are known to periodically oscillate and found to peak during G2/M or mitosis, such as genes from mitotic exit network (dbf2, mob1, bub2, and tem1), FEAR network (apc1, cdc20, and cdc5), spindle positioning (kar1), budding (bud4, bud8, and igg1), and chromosome segregation (nuf2) were chosen to analyze their transcript levels. The contribution of Cdc15 in regulating transcription was measured by checking the transcript levels of the selected mitotic genes using quantitative RT-PCR in the conditional mutant Cdc15-2 at permissive (25 °C) and non-permissive (37 °C) temperatures. The mitotic CTD phosphatase (Cdc14-1) was used as a control to measure and compare the effect during asynchronous, telophase arrest, and G2/M arrest cells (Fig. 4, A–C). The exit from mitosis is prevented at non-permissive temperature for Cdc15-2 and Cdc14-1, and hence the resulting arrest of cells in the telophase would ensure that the observed changes, if any, are limited to mitosis only. The data were normalized against WT cells grown similarly as other cells (described under “Experimental Procedures”). The conditional mutation (Cdc15-2 and Cdc14-1) had no effect on the expression of the selected genes in the asynchronous condition at the permissive temperature (Fig. 4A). However, the non-functional Cdc15 significantly influenced the expression of these genes.

FIGURE 2. CTD is phosphorylated by Cdc15 during mitosis. A–C, Western blotting analysis of cell extract where the cells were arrested at a non-permissive temperature in telophase (Cdc15-2) and released at permissive temperature and probed for the presence of Ser(P)-5 (A), Ser(P)-2 (B), and Rpb3 (C) in mitosis. The prestained molecular mass marker (Puregene PG-PMT2922) was used to assign molecular mass to the detected bands and is marked with arrows. The phosphorylation intensity of the spots was quantified using ImageJ software, and the data resulting from the triplicate blots were analyzed using GraphPad Prism version 5.01. D–F, a similar comparison of the change in CTD phosphorylation in case of control Cdc14-1 mutant cells is also done. Cdc15-2 and Cdc14-1 cells grown at 25 °C and were shifted to 37 °C for 3 h to reach a telophase arrest. The cells were then released into a fresh YPAD medium and grown for an additional 3 h at 25 °C to relieve from mitotic arrest. α-Tubulin was used as a loading control.
**FIGURE 3. Phenotypic analysis of asynchronous and arrested cells.** A, the growth of serially diluted asynchronous wild type and telophase arrested (Cdc15-2, Cdc5-1, and Cdc14-1) strains are shown. The yeast cell suspensions starting with undiluted ($1 \times 10^7$) and 10-fold serially diluted cultures up to $1 \times 10^2$ were placed on solid medium and incubated for 3 days at 18, 25, 30, and 37 °C. B, the morphology of asynchronous wild type, synchronous G$_2$/M (nocodazole), and telophase arrested (Cdc15-2, Cdc5-1, and Cdc14-1) strains are analyzed in Nikon Eclipse E100 light microscope under high magnification (100×).

**FIGURE 4. Cdc15 influences transcription in mitosis.** A, the transcript level of the genes was compared in Cdc15-2 and Cdc14-1 conditional mutants. The cells grown at 25 °C were harvested for RNA isolation prior to shifting at 37 °C for the subsequent experiments. Transcript levels relative to act1 are shown (means ± S.E., n = 3). B, the change in the transcript level of genes arrested in telophase was compared for Cdc15-2 and Cdc14-1. The cells grown at 25 °C were shifted to 37 °C for 3 h to reach a telophase arrest. Transcript levels relative to act1 (endogenous control) are shown for each gene (means ± S.E., n = 3). C, the change in the transcript level of genes arrested in telophase (Cdc15-2 and Cdc14-1) was compared relative to the G$_2$/M. Transcript levels relative to act1 are shown (means ± S.E., n = 3). The transcript level of rpb3, gapdh, h2a, and U2 snRNA was also examined in similar conditions (A–C, right panels), which served as a positive control for the recruitment of RNAPII, housekeeping gene, S phase marker, and a snRNA gene, respectively. The data were analyzed using GraphPad Prism version 5.01. One-way or two-way ANOVA with Bonferroni’s post-tests was used to determine significant differences. Statistical significance was determined by p values <0.05 (*), <0.01 (**), or <0.001 (***). ns represents non-significant.
during telophase arrest at the non-permissive temperature (Fig. 4B). The significant change in the transcript levels in Cdc15-2 cells suggests the role of Cdc15 as a CTD kinase during mitosis. Because the expression of the selected genes oscillates during mitosis, we further tested whether the Cdc15 affects transcription in G2/M (Fig. 4C). It is expected that the genes whose expression peaks at a certain point of cell cycle will have an expression above the basal level around the peak region. Here the data relative to the WT cells grown simultaneously and arrested in G2/M were analyzed. The significant changes observed in the relative expression of genes further support the influence of Cdc15 in regulating transcription during mitosis. To further check whether the observed decrease in transcript level is not because of the defect in the recruitment of RNAPII, we examined the transcript occupancy of rpb3 in similar conditions. The other controls used were the housekeeping gene (gapdh), S phase marker (h2A), and a snRNA gene. The relative change in the transcript level of rpb3 and other genes was insignificant during permissive and non-permissive temperature for asynchronous and arrested cells in the similar conditions (Fig. 4, A–C, right panels). These results suggest the influence of Cdc15 in the mitotic gene expression.

The WT cells cannot be arrested in telophase of mitosis. Furthermore, some population (5–10%) of the asynchronous WT cells may be in the telophase at a given point of time, whereas in case of Cdc15-2 or Cdc14-1 cells (at non-permissive temperature), more than 90% cells remain at telophase. Because of this, a direct comparison between the Cdc15-2 or Cdc14-1 and the wild type cells is not feasible. To circumvent and further support our result, we compared the changes in transcription using another control Cdc5 at similar conditions (Fig. 5). Cdc5 is a mitotic kinase (do not phosphorylates Ser-2 and Ser-5) and an essential component of FEAR complex and required for the function of the mitotic exit network. Like Cdc15-2 and Cdc14-1, Cdc5-1 is also derived from the same background (W303) and belongs to the same mating type a. The significant changes were observed in the majority of the genes when the transcript levels were compared between Cdc15-2 and Cdc5-1 at similar conditions during mitosis and G2/M as described for Fig. 4 (Fig. 5, B and C). The observed insignificant changes in case of mob1, cdc20, kar1, ige1, and cdc28 suggest the indirect role of Cdc5 in influencing the expression of these genes.

Cdc15 Is a CTD Kinase for Transcription during Mitosis—
The effect of the conditional mutant (Cdc15-2, Cdc14-1, and Cdc5-1) on the complete transcription of a gene is expected to give better insight into the role of Cdc15 as a CTD kinase in mitosis. cld2, the cyclin partner of cdc28, is a major mitotic cluster gene that plays an important role in the cell progression.
through mitosis and at the START of G1 (20, 33, 34). The clb2 cluster is self-regulatory and is essential for full transcription activation of its own promoter and hence is proposed to follow a positive feedback loop (35). The human homolog of clb2 (codes for cyclin B1) has been reported to completely transcribe in mitosis by RNAPII (36), and hence we chose the gene to see the effect of Cdc15 and Cdc5 on its complete transcription and the prevailing CTD phosphorylation pattern. The role of Cdc15 in regulating transcription of clb2 was measured at similar conditions as described above for Fig. 4. The Cdc15-2 significantly influenced transcription of clb2 in the arrested cells when compared against either Cdc14-1 (Fig. 6A) or Cdc5-1 (Fig. 6B). We

FIGURE 6. Cdc15 regulates transcription of Clb2 in mitosis. A, the transcript level of clb2 was compared in Cdc15-2 and Cdc14-1 cells. The conditional mutant cells show no effect on the transcript level of clb2 in the asynchronous condition (left panel). The non-functional Cdc15-2 affects the expression of clb2 in the telophase arrest (middle panel) or when compared the telophase arrest relative to G2/M (right panel). Transcript levels relative to act1 are shown for each gene (means ± S.E., n = 3). The cells grown at 25 °C were harvested for RNA isolation prior to shifting at 37 °C for the subsequent experiments. The cells grown at 25 °C were shifted to 37 °C for 3 h to reach a telophase arrest. B, the transcript level of clb2 was compared in Cdc15-2 and Cdc5-1 arrests. The conditional mutant cells show no effect on the transcript level of clb2 in the asynchronous condition (left panel). Cdc15-2 affects the expression of clb2 in the telophase arrest (middle panel) or when compared the telophase arrest relative to G2/M (right panel). The transcript levels relative to act1 are shown (means ± S.E., n = 3). C, the ChIP analysis of Ser-5 phosphorylation at the five different regions (promoter to 3′ end) at clb2 gene. The effect of cdc15 inactivation at telophase in comparison with G2/M arrest by nocodazole (Cdc15-2 versus G2/M) was analyzed without the effect of temperature induced variation (37 °C/25 °C). The relative fold change of Cdc15 occupancy in the G2/M (WT(NOC)) and telophase (Cdc15-2) arrested cells are also shown. D, the ChIP analysis of Ser-2 phosphorylation at the five different regions (promoter to 3′ end) of Clb2 in Cdc15-2 versus G2/M cells at 37 °C/25 °C. Mock IP values were subtracted from the IP values to generate the fold increase in signals using the 2^ΔΔCT method. The data were analyzed using GraphPad Prism version 5.01. The relative fold change of Cdc15 occupancy in the WT(NOC) and arrested cells is also shown.
further looked at the effect of Cdc15 inactivation on Ser(P)-5 and Ser(P)-2 occupancy over the entire gene length (promoter to termination) of clb2. The Ser(P)-5 occupancy over the clb2 gene was checked for Cdc15-2 in comparison with nocodazole-arrested cells (G2/M arrest). The result shows comparatively reduced Ser(P)-5 occupancy at promoter in case of Cdc15-2, which suggests the influence of the kinase on phosphorylating Ser-5 of CTD during transcription initiation (Fig. 6C). A similar comparison with respect to the Ser(P)-2 occupancy was also checked for clb2, and the result shows a reduced Ser(P)-2 level toward 3′ end only (Fig. 6D). The observation of reduced transcription of clb2 accompanied by reduced Ser(P)-5 occupancy at the promoter and of Ser(P)-2 occupancy at the 3′ end of the gene with no significant change of Ser(P)-2 at the promoter suggests that the prevailing phosphorylation occupancy and envisaged transcription regulation during mitosis are influenced by Cdc15 (Fig. 6). The Cdc15 occupancy at clb2 for Ser(P)-5 and Ser(P)-2 further supports the role of the kinase in CTD phosphorylation during mitosis (Fig. 6, C and D). Unlike the interphase transcription, where Kin28 phosphorylates Ser-5 at the promoter (needed for transcription initiation) and Bur1/Ctk1 phosphorylates Ser-2 toward 3′ end of the genes (needed for transcription elongation and termination), Cdc15 alone phosphorylates both Ser-5 and Ser-2 at the respective positions of clb2 to accomplish its active transcription during mitosis.

The analysis of transcription profiles on the genome-wide strand-specific tiling arrays in Cdc14-1 and Cdc15-2 arrested cells mostly shows a similar overlapping profile. The Cdc14 acts directly on RNAPII CTD phosphorylation (Ser-2 and Ser-5) and causes silencing defects at the Y element of the right arm of chromosome IV (TEL4R-Y). The Cdc14 inactivation (Cdc14-1) increased the Ser(P)-2 and Ser(P)-5 at TEL4R-Y, whereas the Cdc15 inactivation (Cdc15-2) decreased such phosphorylations (16). The analysis of the above studies further hints at Cdc15 as a potential mitotic CTD kinase, because the release of Cdc14 and activation by Cdc15 in these cases is primarily after anaphase. We further checked the effect of Cdc15 on the expression of TEL4R-Y. The insignificant decrease in the transcript level in case of asynchronous cells, but a significant decrease in the transcript level for the arrested cells, suggests a role of Cdc15 in the expression of TEL4R-Y (Fig. 7, A and B).

**Discussion**

The transcription initiation is mediated through the binding of general transcription factors and RNAPII to the gene promoter. Further, the Ser-5 of CTD is phosphorylated by Kin28/Cdk7, resulting in the release from the preinitiation complex and transcription initiation. The subsequent CTD phosphorylation at Ser-2 by other kinases leads to further transcription processes. Of late the Ser-7 was shown to be phosphorylated by Kin28 and Bur1, and their direct role was mainly envisaged for the snRNA transcription (1–4). In the presence of an inactive kinase subunit CAK of TFIIH during mitosis, as well as at non-permissive temperature, a transcription is inevitable without the Ser-5 phosphorylation by other kinases. Here we show that Cdc15 phosphorylates both the Ser-5 and Ser-2 of CTD required for the accomplishment of transcription during mitosis. The phosphorylation of CTD in mitosis by Cdc15 is also supported by the fact that the inhibition (Cdc15-as) or inactivation (Cdc15-2) of Cdc15 leads to the significant reduction of CTD phosphorylations. The slightly reduced but insignificant changes in the Ser(P)-5 level for Cdc14-1 at non-permissive and permissive temperature signifies the importance of CTD phos-
phorylation cycle for transcription during mitosis. The significant reduction in transcript levels observed in the telophase arrest or in comparison with G2/M suggests the crucial role of Cdc15 in phosphorylating CTD to accomplish transcription during mitosis. To understand the placement, removal, and roles of CTD modifications, it is important to know the CTD modification enzymes and to characterize the full range of their substrates. The finding of Cdc15 as a potential CTD kinase during transcription in mitosis will further invoke research in understanding the underlying mechanism of transcription.

**Experimental Procedures**

**Plasmids and Strains**—Yeast strains and plasmids used in this study are listed in Table 2. All the TAP tag yeast kinase and control (BY4741) strains were purchased from Open Biosystems (Huntsville, AL).

**Cloning and Expression of CTD Mutants**—Sequences for S25S5A7 (A7), A25S5A7 (A27), S2A5A7 (A57), and A2A5A7 (A3) and 14 CTD mutants were codon optimized for synthesis by integrated DNA Technologies (Coralville, IA). Subcloning of these mutants from pDTSmart-Amp vector to pGEX-4T1 was performed following BamHI and HindIII digestion of vectors; the resulting inserts from pDTSmart-Amp vector were ligated into pGEX-4T1 vector cut with the same enzymes. *Escherichia coli* DH5-α competent cells were used to transform the plasmid constructs and screened for positive clones. The resulting clones were expressed in *E. coli* C41 strain and purified as described previously (5).

**Cell Culture and Synchronization**—The strains Cdc15-2, Cdc15-1, Cdc14-1, Cdc5-1, and BY4741 (WT grown in triplicate) were grown in 1× YPAD medium at 25 °C with shaking (200 rpm) at A600 ~0.35–0.4. The fractions of each culture were collected for lysate preparation and ChIP. The remaining cultures were shifted to non-permissive temperature (37 °C) and grown for an additional 3 h. Simultaneously prior to shifting at 37 °C, rest two cultures of BY4741 were respectively treated with nocodazole (15 μg/ml) and DMSO. Subsequent to the growth arrest, three fractions of the culture (treated with DMSO or nocodazole) were harvested for (i) lysate preparation, (ii) RNA isolation, and (iii) ChIP assay. The cells were then released from mitotic arrest and harvested for lysate preparation. For ChIP analysis, an additional step of formaldehyde fixing (1.4 ml of 37% formaldehyde for 20 min, followed by 5 min of quenching with 2 ml of 2.5 M glycine) was performed. Similarly, Cdc15-as1 cells were arrested in G2/M with nocodazole (15 μg/ml) in 1× YPAD at 30 °C and then released in nocodazole-free medium containing either DMSO or 1-NAPPP1 (10 μM). The cells were grown for an additional 1.5 h at 30 °C and harvested for the lysate preparation. The samples were washed, respectively, with 1× PBS for the lysate preparation, DEPC water for the RNA isolation and 1× TBST for ChIP. After complete removal of residual buffers, the cells were immediately snap frozen in liquid nitrogen and stored at −80 °C until the experiment.

**TAP-tagged Protein Purification and in Vitro Kinase Assay**—All the TAP-tagged kinases were purified and assayed using an equal amount of the purified kinases as described previously (29). Briefly, *S. cerevisiae* cells having TAP tag at the C terminus of kinases were grown to A600 ~1.5 and collected at 5836 × g for 10 min at 4 °C. The cells were washed with 25 ml of MilliQ. Pellet was resuspended in 25 ml of TAP extract buffer (40 mM Hepes-KOH, pH 7.5, 10% glycerol, 350 mM NaCl, 0.1% Tween 20, and fresh 1 μg/ml pepstatin A, 2 μg/ml leupeptin, 0.5 mM EDTA, and 1 mM PMSF) and lysed by French press at 4 °C. The lysate was treated with 100 units of DNaseI for 10 min at room temperature, and the extract was clarified by centrifugation at 20,028 × g for 1 h at 4 °C. 50 μl of IgG-Sepharose beads were washed with 10 ml of TAP extract buffer and added to the clarified supernatant and incubated on the rotating platform overnight at 4 °C. The extract resin suspension was kept on ice for 15 min to allow beads to settle down and separate the supernatant from the beads. The beads were washed with 20 ml of TAP extract buffer followed by washing with 3 ml of TEV cleavage buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 10% glycerol, and freshly added 1 mM PMSF; 2 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM DTT). The beads were resuspended in 100 μl of TEV cleavage buffer, and 1.0 μl (10 units) of AcTEV protease (Invitrogen) was added followed by incubation on a rotating platform for overnight in the cold room. The TEV-cleaved protein was eluted by gravity flow at 4 °C.

The assay was performed in 25 μl of reaction volume at 25 °C for 1 h in buffer containing 20 mM Hepes-KOH, pH 7.5, 2.5 mM EGTA, 15 mM magnesium acetate, 0.8 mM ATP, 10% glycerol, and 1× protease and 1× phosphatase inhibitor cocktails.
tein was estimated using Bradford reagent (Sigma). 200 mg of lysate were clarified by centrifugation at 16,200 g for 45 min. RNA yield and purity were evaluated with the NanoDrop spectrophotometer (NanoDrop1000; Thermo Scientific), whereas RNA quality was examined by denaturing gel electrophoresis. RNA Isolation and Evaluation of Mitotic Transcripts by Real Time PCR—Total RNA was extracted by using the RNeasy mini kit (Qiagen) following the manufacturer’s instructions with minor modifications. Briefly, frozen S. cerevisiae cell pellets were thawed and resuspended in 1 ml of RNeasy lysis buffer (Qiagen) and transferred to a 2-ml microcentrifuge tube containing Magna Lyser beads (Roche Life Science). Tubes were immediately processed for cell disruption in the bead beater for six cycles of 40 s each with cooling on ice for 1 min. The remaining steps were performed according to the manufacturer’s instructions. To remove trace amounts of genomic DNA, RNA samples were treated with DNasel (Thermo Fisher Scientific) for 45 min. RNA yield and purity were evaluated with the Nanodrop spectrophotometer (NanoDrop1000; Thermo Scientific), whereas RNA quality was examined by denaturing gel electrophoresis.

A small aliquot from the reaction mix was spotted directly onto PVDF membrane and further processed as standard dot blotting. For the assay, 1:500 dilution of primary rat IgG (anti-TAP tag; Pierce), and 1:10,000 secondary HRP anti-rat and anti-rabbit IgG (Santa Cruz) antibodies were used. Chemiluminescent signals were detected using Image Quant LAS 4000 instrument (GE Healthcare).
1 μg of total RNA was reverse transcribed in a final volume of 20 μl to generate first strand cDNA by using random primers, RevertAid H M-MuL V reverse transcriptase provided in the RevertAid H M-Minus First Strand cDNA synthesis kit. Control reactions, lacking reverse transcriptase, were performed for every RNA sample.

Primers used in this study are listed in Table 2. Real time PCRs were accomplished using the Light Cycler® 480 II system in a total volume of 10 μl, using SYBR green-based detection. PCR amplification conditions comprise an initial cycle of denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at optimal temperature for 15 s, and extension at 72 °C for 15 s. Fluorescence was measured at the end of the annealing/extension step. The reactions were run in triplicate for each gene, and the specificity of the PCR products was verified by gel electrophoresis and melting curve analysis. The expression values were calculated using the 2^(-ΔΔCT) method (37). The p values were calculated using one-way or two-way ANOVA with Bonferroni’s post-tests to determine the significant difference between mutants. All values obtained are the means ± S.D. of triplicate experiments in three biological replicates.

ChIP-qPCR—ChIP was carried out as described previously (28) with minor modifications. Briefly, 25 μl of whole cell extract (equivalent to 250 μg) was diluted to 500 μl with lysis buffer. This diluted extract was incubated overnight at 4 °C with appropriate antibody (50 μl of 3E8 and 3E10, 10 μl of anti-Rpb3). Following elution 2 μl of RNase A (Fermentas) was added and incubated at 37 °C for 1 h; then a further 5 μl of proteinase-K (Fermentas) was added and incubated at 65 °C for ~16 h. Immunoprecipitated DNA samples were subjected to qPCR for 40 cycles of amplification, and quantification of different fragments of mitotic gene clb2 was performed as described above. Mock IP (i.e. no antibody control) values were subtracted from the IP values to generate the fold increase in signals using 2^(-ΔΔCT) method. The sequences of primers used for ChIP-qPCR study are listed in Table 3.

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