Swe1 regulation and transcriptional control restrict the activity of mitotic cyclins toward replication proteins in *Saccharomyces cerevisiae*

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Cyclin-dependent kinases (CDKs) drive the cell cycle through the phosphorylation of substrates that function in genome duplication and cell division. The existence of multiple cyclin subunits and their distinct cell cycle-regulated expression suggests that cyclins impart unique specificities to CDK–substrate interactions that are critical for normal cellular function. This study shows that the combination of early cell cycle expression and deletion of the CDK inhibitor Saccharomyces Wee1 (Swe1) enables the mitotic B-type (Clb) cyclins Clb2, Clb3, and Clb4 of *Saccharomyces cerevisiae* to initiate S phase with similar effectiveness as the S-phase cyclin Clb5. Although *in vivo* analysis indicates preferential phosphorylation of a replication substrate by Clb5-Cdk1, this difference is relatively minor compared with the impact of transcriptional control and Swe1 regulation. Indeed, early expressed Clb2-Cdk1 can activate all essential Clb-Cdk substrates in a strain lacking all other Clbs and Swe1. Thus, Swe1 regulation and expression timing are key mechanisms that sequester the broad activity of Clb2-Cdk1 from critical substrates. Furthermore, the ability of Swe1 to inhibit the activity of different B-type cyclins in replication initiation correlates with the normal expression timing of those cyclins, with no apparent *in vivo* inhibition of Clb5 and Clb6, moderate inhibition of Clb3 and Clb4, and strong inhibition of Clb2. Hence, Swe1 appears to reinforce the temporal activity of cyclins established through transcriptional control. The conserved nature of CDK function suggests that similar mechanisms regulate CDK specificity in multicellular organisms.

B-type cyclin | cyclin-dependent kinase | protein evolution | replication origin | Swe1

In eukaryotic cells, cyclin-dependent kinase (CDK) controls the cell cycle by phosphorylating different substrates at different times during the cell cycle. CDK consists of a kinase whose function is stimulated by association with a "cyclin," whose expression is cell cycle-regulated. In *Saccharomyces cerevisiae*, G_1 cyclins (Cln1, Cln2, and Cln3) and B-type cyclins (Clb)1–6 sequentially activate a single catalytic subunit, Cdk1 (Cdc28) (reviewed in ref. 1). The six B-type cyclins are thought to have evolved from a single ancestral B-type cyclin that could carry out all cell cycle functions (2). Gene duplications of the ancestral cyclin apparently have allowed for the evolution of functionally diverse cyclins, as evidenced by their different patterns of cell cycle expression and different phenotypes resulting from deletion of individual or multiple cyclin genes.

The *CLB* genes are expressed periodically, in pairs, with *CLB5* and *CLB6* transcripts peaking at the beginning of S-phase (3–5), *CLB3* and *CLB4* transcripts peaking in late S-phase (6, 7), and *CLB1* and *CLB2* transcripts peaking during mitosis (5–8). Each cyclin pair drives distinct cell cycle events that coincide with its time of expression, such as DNA replication (Clb5 and Clb6), spindle morphogenesis (Clb3 and Clb4), and mitosis (Clb1 and Clb2) (reviewed in ref. 1). Nevertheless, functional redundancy exists among these cyclins, because none is essential, and some double and triple deletions are viable. For example, *clb5*Δ *clb6*Δ mutant cells are viable, indicating that one or more of the remaining Clbs is capable of activating replication origins (3, 4). Similarly, *clb3*Δ *clb4*Δ mutant cells are viable (6, 7), and constitutive overexpression of *CLB1* in cells lacking all B-type cyclins maintains viability (9). These results indicate that certain B-type cyclins can substitute for certain others (at least for vital functions) and suggest that much of their functional divergence lies in their different expression patterns rather than substrate specificities.

Despite this functional overlap, genetic analyses also suggest that functional specificity exists (reviewed in ref. 10). For example, deletion of *CLB5* and *CLB6* becomes lethal in the absence of *CLB3* and *CLB4*, suggesting that Clb3–Clb6 carry out at least one essential function (probably DNA replication), which cannot be performed by normal levels of Clb1 and Clb2. Detailed studies of different cyclins' abilities to activate replication origins also argue for functional differences. In the absence of Clb5 and Clb6, which normally activate replication origins, initiation of S-phase is delayed significantly compared with wild type, indicating that origin activation occurs when expression of one or more of the "mitotic" cyclins (Clb1–Clb4) occurs later in the cell cycle (4). However, earlier expression of *CLB2* or *CLB4* (by using the *CLB5* promoter) did not appreciably advance S-phase entry in *clb5*Δ *clb6*Δ cells (11, 12). The failure of early expressed Clb2 to advance the time of S-phase entry was not due to a failure to properly accumulate Clb2-associated kinase activity (by using histone H1 as the substrate), which occurred with similar kinetics and to equivalent or higher levels than Clb5-associated kinase activity in wild-type cells (11). These findings have led to the conclusion that the functional specificity of B-type cyclins derives from intrinsic differences in their abilities to target Cdk1 activity toward replication factors. An alternative, although not exclusive, explanation for the inability of Clb2 or Clb4 to efficiently drive S-phase entry, which has not been examined, is the possibility of differential regulation of specific Clb-Cdks by posttranscriptional mechanisms.

In eukaryotic cells, the activity of Cdk1 is regulated by a cycle of phosphorylation and dephosphorylation of two highly conserved residues (T14, Y15) (reviewed in ref. 13). Members of the Wee1 kinase family phosphorylate Cdk1. Phosphorylation inhibits the activity of Cdk1, and in some organisms, is a critical mechanism of DNA damage and replication checkpoint pathways that delay mitosis (14, 15). Dephosphorylation of Cdk1 by a Cdc25 phosphatase family member is required for reactivation of Cdk1 and mitotic progression. In *S. cerevisiae*, Cdk1 phosphorylation of the analogous tyrosine (Y19) occurs during normal, unperturbed cell cycles during the S and G_2 periods and depends on Saccharomyces Wee1 (Swe1) (16–18). Although DNA damage and replication checkpoints in *S. cerevisiae* block mitosis through alternative mechanisms that do not require inhibitory phosphorylation of Cdk1-Y19 by Swe1 (16, 17), Swe1-mediated inhibition of Cdk1 acts in a cell size or morphogenesis checkpoint to delay mitosis in response to defects

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Abbreviations: CDK, cyclin-dependent kinase; Clb, B-type cyclin; Swe1, Saccharomyces Wee1.

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in growth or bud formation (19, 20). This mechanism appears to operate until the bud has reached a critical size, presumably sufficient for mitotic entry (19). Overexpression of Swe1 inhibits mitotic functions attributed to Clb2, such as spindle elongation, but has not been shown to inhibit DNA replication, which is normally carried out by Clb5 and Clb6, suggesting that the action of Swe1 is cyclin-specific (18).

In this study, we have examined whether Swe1 regulates the ability of the mitotic cyclins, Clb2, Clb3, and Clb4, when expressed early in the cell cycle, to perform the function of the S-phase cyclins, Clb5 and Clb6. Our findings support the view that differential expression timing and cyclin-specific inhibition by Swe1 are key mechanisms that diversify the functions of B-type cyclins in *S. cerevisiae*.

Materials and Methods

Plasmid and Strain Constructions. pΔswe1-URA3 was constructed by four-way ligation of a 400-bp PCR-amplified XbaI-XhoI 5′ *SWE1* fragment, a 500-bp PCR-amplified BamHI-EcoRI 3′ *SWE1* fragment, a 1.2-kb XhoI-BamHI *URA3* fragment, and XbaI-EcoRI digested pBluescript-KS+. pC5C2–3NF (*clb5*::*CLB2*) and pCLB5-CLB4 have been described in refs. 11 and 12. The *CLB3* ORF was PCR-amplified with NotI-EcoRI ends, sequenced to confirm that no mutations were introduced, and used to exactly replace the *CLB2* ORF in pC5C2–3NF, yielding plasmid pCLB5-CLB3. The 1.4-kb KpnI-SalI fragment of pBS-SLD2–9Myc-LEU2 was inserted into pRS404 cut with the same enzymes to yield p404-SLD2–9Myc.

For unmarked insertion of *CLB2*, *CLB3*, or *CLB4* at the *CLB5* locus, pC5C2–3NF digested with XhoI, pCLB5-CLB3 digested with ClaI and SacII, or pCLB5-CLB4 digested with HindIII, was transformed into a *clb5*Δ::*URA3* host; transformants were selected on 5-fluoroorotic acid and confirmed by PCR. Epitope-tagging and gene deletions were constructed as described in ref. 21 with the following exceptions: *SWE1* was deleted in some cases by using pΔswe1-URA3 digested with NotI and EcoRI; *CLB2* was HA-tagged with plasmid pDK82B(TRP) digested with BglII; *CLB5* was HA-tagged with plasmid pKHA3-CLB5 digested with Bsu36I; *SLD2* was HA-tagged with plasmid p404-SLD2–9Myc digested with MscI; and *CLB5* and *CLB6* deletions have been described in ref. 4. All other strains were constructed by standard mating and spore dissections. All strains were derived from W303 and are described in Table 2, which is published as supporting information on the PNAS web site.

Yeast and Other Methods. Yeast extract-peptone-dextrose medium was used for all experiments except for induction of sporulation. Spore analysis has been described in ref. 22. Two-dimensional agarose gel electrophoresis and DNA content analyses have been described in ref. 23, except that quantification of the proportion of cells with a defined DNA content was performed by using IMAGEQUANT software (Becton Dickinson). Sld2 protein was separated on 10% (75:1) SDS polyacrylamide gels. For analysis of Clb-associated kinase activity, protein isolation and H1 kinase assays were performed essentially as described in ref. 11 except that we used 5% as much immunoprecipitated enzyme.

Results

Swe1 Inhibits Replication Initiation by Clb2-Cdk1 During S and G_2. To determine whether Swe1 regulates the potential activity of mitotic cyclins in the early cell cycle, we deleted *SWE1* in *clb5*Δ *clb6*Δ cells expressing *CLB2* from the *CLB5* promoter (*clb5*::*CLB2 clb6*Δ) and monitored replication by assaying DNA content of cells released synchronously from G_1 arrest (Fig. 1*A*). In *clb5*Δ *clb6*Δ cells, replication began between 60 and 72 min, much later than in *clb6*Δ cells, in which Clb5 initiates replication between 12 and 24 min. Early expression of *CLB2* in *clb5*Δ *clb6*Δ cells (*clb5*::*CLB2 clb6*Δ) advanced S-phase entry by ≈12 min. Deletion of *SWE1* significantly advanced replication initiation in cells expressing *CLB2* early (Fig. 1*A*). S-phase began between 24 and 36 min in *clb5*::*CLB2 clb6*Δ *swe1*Δ cells, ≈36 min sooner than in the *clb5*Δ *clb6*Δ cells, and only ≈12 min behind *clb6*Δ cells. The duration of S-phase was comparable in all strains. There was no effect of *SWE1* deletion in *clb6*Δ cells, indicating that Swe1 does not inhibit the function of Clb5 in replication initiation. Because early S-phase entry depends on early expressed Clb2 in addition to *SWE1* deletion, the data suggest that S-phase entry fully depends on Clb2 and that Clb3 and Clb4 did not contribute. To strengthen this conclusion, we analyzed the timing of S-phase entry in cells lacking *CLB3* and *CLB4* (construction of this strain is described below). The time of S-phase entry of *clb5*::*CLB2 clb3*Δ *clb4*Δ *clb6*Δ *swe1*Δ cells was indistinguishable from that of *clb5*::*CLB2 clb6*Δ *swe1*Δ cells, indicating that Clb2 can efficiently stimulate the origin initiation independently of Clb3-Clb6 (Fig. 1*A*).

DNA content analysis of unsynchronized cultures was consistent with these results. Compared with *clb6*Δ cells, *clb5*Δ *clb6*Δ cells show an increased number of cells with unreplicated DNA content because of delayed S-phase entry (Fig. 1*A*). Whereas early expression of *CLB2* or deletion of *SWE1* did not significantly reduce the proportion of cells in G_1, the combination of both reduced the population of these cells significantly, consistent with the accelerated onset of S-phase (Fig. 1*A*). Thus, Clb2 can effectively activate replication origins but is restricted from doing so during the normal S-phase period by its normal transcription timing and the inhibitory action of Swe1.

Swe1 likely acts directly to inhibit early expressed Clb2-Cdk1 function through inhibitory phosphorylation of Cdk1-Y19. To examine directly the importance of Cdk1-Y19 phosphorylation, we expressed a nonphosphorylatable form of Cdk1 (Cdc28-y19F) (16, 17), which should phenocopy *SWE1* mutation in cells expressing *clb5*::*CLB2*. In fact, early expression of Clb2 and Cdc28-y19F advanced S-phase entry of *clb5*Δ *clb6*Δ cells by at least 24 min (Fig. 1*B*). The slightly smaller effect of Cdc28-y19F than deletion of *SWE1* is consistent with reports that part of the Cdk1 inhibitory effect of Swe1 is independent of Y19 phosphorylation (24).

Although early expression of Clb2 in *clb5*Δ *clb6*Δ *swe1*Δ cells drove much earlier S-phase entry, it still was delayed ≈12 min compared with *clb6*Δ and *clb6*Δ *swe1*Δ cells, in which Clb5 stimulates S-phase entry (Fig. 1*A*). This result does not appear to reflect a difference in the time of cell cycle entry (start) because the time of bud emergence in *clb5*::*CLB2 clb6*Δ *swe1*Δ cells was indistinguishable from *clb6*Δ *swe1*Δ cells (data not shown). It did not appear to reflect instability of Clb2 in the early cell cycle, perhaps due to Cdh1-dependent degradation, because early expression of stabilized Clb2 lacking its destruction box drove replication initiation in *clb5*Δ *clb6*Δ *swe1*Δ cells with similar kinetics as Clb2 (data not shown). Increasing the dosage of early expressed Clb2 (by using a low-copy, plasmid-borne *clb5*::*CLB2* in addition to the integrated copy) did not further accelerate S-phase entry either (data not shown). The slight difference in the ability of Clb2 to stimulate replication versus Clb5 may reflect inherent differences in their substrate specificities.

Clb3 and Clb4 Can Stimulate Replication Initiation in the Presence of Swe1. We also tested whether Swe1 blocks the function of early expressed *CLB3* and *CLB4.* In contradiction to a previous report that early expression of *CLB4* in place of *CLB5* did not effectively activate origins (12), we observed that early expression of Clb4 advanced S-phase by ≈24 min (Fig. 1*C*). Deletion of *SWE1* further advanced S-phase in *clb5*::*CLB4 clb6*Δ cells by ≈12 min. Early expression of Clb3 in *clb5*Δ *clb6*Δ cells also advanced replication initiation, in this case by ≈36 min, which was similar to the effect of Clb2 or Clb4 early expression in the absence of Swe1 (Fig. 1 *A* and *C*). Deletion of *SWE1* in *clb5*::*CLB3 clb6*Δ cells advanced S-phase by a small (≈6 min) but reproducible degree (Fig. 1*C*). The DNA content of unsynchronized cultures supported these findings, because early expression of Clb3 or Clb4 with and, to a greater degree, without Swe1 decreased the proportion of cells with

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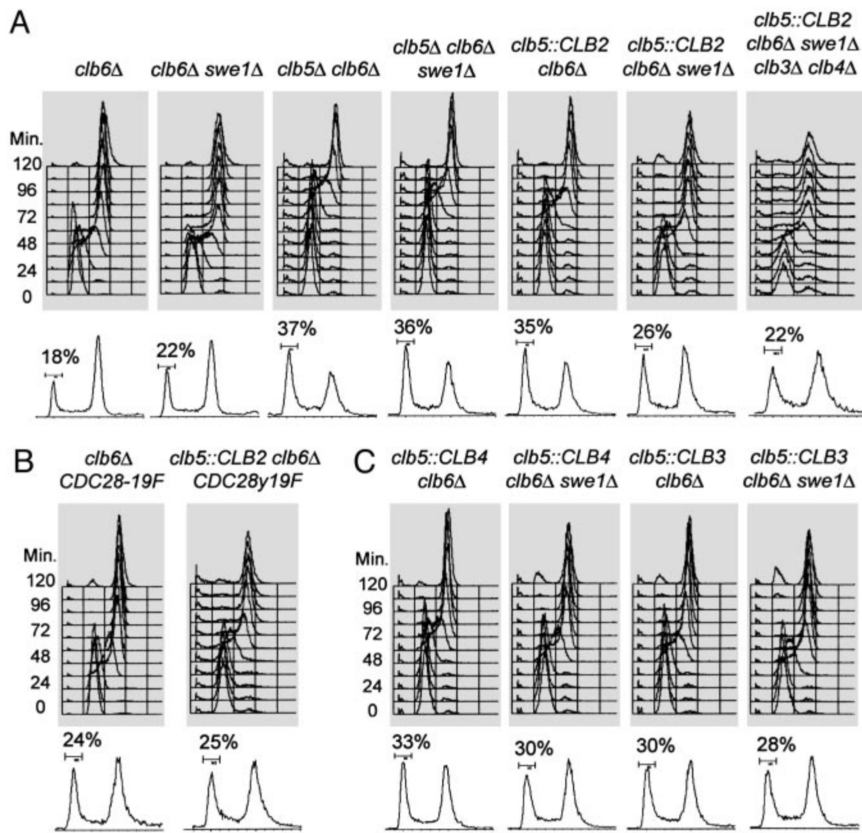

Fig. 1. Swe1 inhibits the function of early expressed Clb2 in replication initiation. DGy221 (*clb6*Δ), FHy136 (*clb6*Δ *swe1*Δ), DGy228 (*clb5*Δ *clb6*Δ), FHy133 (*clb5*Δ *clb6*Δ *swe1*Δ), FHy116 (*clb5*::*CLB2 clb6*Δ), FHy134 (*clb5*::*CLB2 clb6*Δ *swe1*Δ), FHy209 (*clb5*::*CLB2 clb3*Δ *clb4*Δ *clb6*Δ *swe1*Δ), FHy225 (*clb6*Δ *CDC28-y19F*), FHy219 (*clb5*Δ::*CLB2 clb6*Δ *CDC28-y19F*), FHy165 (*clb5*Δ::*CLB4 clb6*Δ), FHy167 (*clb5*Δ::*CLB4 clb6*Δ *swe1*Δ), OAy927 (*clb5*Δ::*CLB3 clb6*Δ), and OAy929 (*clb5*Δ::*CLB3 clb6*Δ *swe1*Δ) cells were blocked in G_1 with α-factor and released at 23°C. Every 12 minutes, samples were collected for DNA content analysis. The lower panels show the DNA content of unsynchronized, logarithmically growing cells, with quantification of the percentage of cells with unreplicated genomes indicated by the marker.

unreplicated DNA content (Fig. 1*C*). Together, these findings demonstrate that mitotic cyclins can effectively target Cdk1 toward essential replication factors. Our results further demonstrate that Swe1 strongly inhibits Clb2, has intermediate activity toward Clb3 and Clb4, and does not inhibit Clb5 or Clb6, at least toward replication substrates in this *in vivo* assay (Fig. 1*A*; for effect on Clb5, compare *clb6*Δ and *clb6*Δ *swe1*Δ, and data is not shown for Clb6).

Late-Firing Replication Origins Can Use Any Clb-Cdk1 for Activation. Next, we examined in more detail the function of mitotic cyclins in origin initiation, particularly late-firing origins. Previous studies have shown that late origin firing is defective in cells lacking Clb5 and that early expression of Clb2 does not compensate for lack of Clb5 (12, 25). However, we suspected that regulation by Swe1 could explain the failure of Clb2 to activate late origins in the previous study. To test this hypothesis, we examined initiation at one early (ARS305) and two late-firing origins (ARS603 and ARS1011) by using 2D agarose gel electrophoresis analysis of replication structures. Efficient activation of all three origins occurred in wild-type cells, indicated by the presence of "bubble" and "large Y" structures (Fig. 2*A*, filled and open arrowheads, respectively). In *clb5*Δ cells, early origin firing was efficient; however, late origin firing was inefficient, indicated by an almost complete absence of a bubble arc and corresponding increase in smaller Y structures, which are particularly apparent at the apex of the Y arc (Fig. 2*A*, double arrowhead). Individually, early expression of Clb2 (*clb5*::*CLB2*) or elimination of Swe1 (*clb5*Δ *swe1*Δ) increased only very slightly the efficiency of late origin firing in *clb5*Δ cells (Fig. 2*A*). However, early expression of Clb2 along with deletion of Swe1 (*clb5*::*CLB2 swe1*Δ) significantly increased the frequency of late origin firing in *clb5*Δ cells, indicating that Clb2 is able to activate late-firing origins (Fig. 2*A*, compare the relative intensities of bubble and large Y structures to the ascending Y arc and apex of the Y arc).

The failure of late origin firing in *clb5*Δ cells lengthens S-phase, as demonstrated by the increased proportion of S-phase cells in an unsynchronized culture (Fig. 2*B*). Cultures of *clb5*::*CLB2* and *clb5*Δ *swe1*Δ cells show a similar proportion of S-phase cells as *clb5*Δ cultures, suggesting no rescue of the replication defect by early expressed Clb2 or the deletion of *SWE1* (Fig. 2*B*). However, the combination of early expressed Clb2 and *SWE1* deletion in *clb5*Δ cells decreased the length of S-phase (Fig. 2*B*), consistent with the restoration of late origin firing in *clb5*::*CLB2 swe1*Δ cells (Fig. 2*A*). Thus, Clb2 is able to activate early and late firing replication origins and to complete chromosomal replication at a rate similar to wild-type cells.

Although Clb2 clearly was able to increase late origin firing in *clb5*::*CLB2 swe1*Δ cells, it was not as efficient as in wild-type cells (Fig. 2*A*). We suspected that this result was due to the presence of Clb6 (in *clb5*::*CLB2 swe1*Δ cells), which, like Clb5, initiates S-phase slightly earlier than Clb2 (data not shown). Thus, Clb6 generates replication forks that can replicate through any unfired origins, particularly late-firing origins, and interfere with our ability to observe their activation by Clb2. To address this possibility, we analyzed late origin efficiency in cells lacking Clb6. In *clb5*Δ *clb6*Δ and *clb5*Δ *clb6*Δ *swe1*Δ cells, ARS603 initiated with efficiency similar to wild-type cells; thus, late origin activation can be attributed to one or more of the mitotic cyclins present (Clb1–Clb4) (Fig. 2*C*) (25). In *clb5*::*CLB2 clb6*Δ *swe1*Δ cells, the early S-phase entry wholly depends on Clb2 (Fig. 1*A*); hence, late origin firing in this strain should be attributable to Clb2. Late origin activation in *clb5*::*CLB2 clb6*Δ *swe1*Δ cells was nearly as efficient as in *clb5*Δ *clb6*Δ and *clb5*Δ *clb6*Δ *swe1*Δ cells (Fig. 2*C*, data not shown for ARS1011), indicating that Clb2 could effectively activate late origins. Similarly, early expressed Clb3 can drive late origin activation, because ARS603 activation was efficient in *clb5*::*CLB3 clb6*Δ *swe1*Δ cells, in which S-phase entry fully depends on early expressed Clb3 (Fig. 2*C*). To rule out that Clb3 or Clb4 contributed

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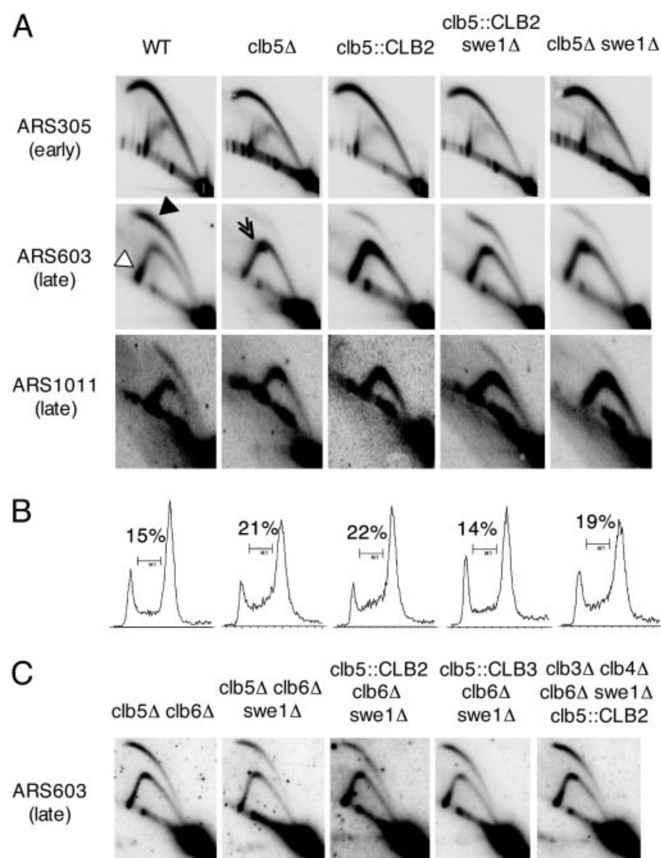

Fig. 2. Clb2 and Clb3 effectively activate early and late origins. Unsynchronized cells of strains OAy470 (WT), DGy226 (*clb5*Δ), FHy119 (*clb5*::*CLB2*), FHy137 (*clb5*::*CLB2 swe1*Δ), FHy138 (*clb5*Δ *swe1*Δ), DGy228 (*clb5*Δ *clb6*Δ), FHy133 (*clb5*Δ *clb6*Δ *swe1*Δ), FHy134 (*clb5*::*CLB2 clb6*Δ *swe1*Δ), OAy929 (*clb5*::*CLB3 clb6*Δ *swe1*Δ), and FHy209 (*clb5*::*CLB2 clb3*Δ *clb4*Δ *clb6*Δ *swe1*Δ) grown at 23°C were subjected to 2D agarose gel electrophoresis analysis (*A* and *C*). (*B*) DNA content of the cells in *A* is shown. The 2D agarose gel electrophoresis was normalized by the intensity of the 1C spot. Filled and unfilled arrowheads show examples of bubble and large fork structures, respectively, which are indicative of origin intitiation. The double arrowhead shows an example of intermediate size forks, which result from passive replication.

to late origin firing by Clb2 in *clb5*::*CLB2 clb6*Δ *swe1*Δ cells, we analyzed cells also lacking *CLB3* and *CLB4*. Clb2 stimulated initiation of ARS603 in *clb3*Δ *clb4*Δ *clb5*::*CLB2 clb6*Δ*swe1*Δ cells as efficiently as in *clb5*Δ *clb6*Δ and *clb5*Δ *clb6*Δ *swe1*Δ cells (Fig. 2*C*). Together with recent data indicating that increased Clb6 dosage can drive late origin firing (22), these data demonstrate that early and late-firing replication origins can use any Clb-Cdk1 for activation (however, Clb1 remains untested).

Phosphorylation of a Critical Replication Factor by Clb2-Cdk1 Is Restricted by Swe1 and Transcriptional Control. To examine directly the ability of Clb2-Cdk1 to target a normal Clb5- and Clb6-Cdk1 substrate, we monitored *in vivo* phosphorylation of Sld2, whose phosphorylation is essential for replication initiation (26). Based on its mobility shift in SDS/PAGE, the timing of Sld2 phosphorylation corresponded with S-phase entry in all strains (Fig. 3). In particular, Sld2 phosphorylation occurred at $\approx$24 min in *clb6*Δ cells and at $\approx$36 min in *clb5*::*CLB2 clb6*Δ *swe1*Δ cells. Swe1 significantly delayed Sld2 phosphorylation in *clb5*::*CLB2 clb6*Δ cells (Fig. 3). Thus, early expressed Clb2-Cdk1 can phosphorylate an essential replication initiation factor, but Swe1 inhibits this activity of Clb2-Cdk1. Interestingly, there are differences in Sld2 phosphorylation by Clb5-

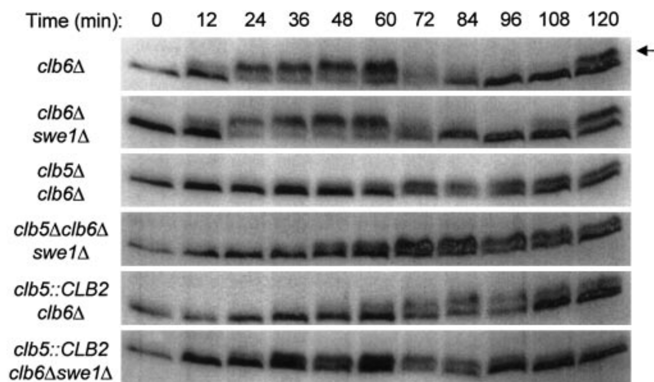

Fig. 3. Swe1 and transcription regulate phosphorylation of Sld2 by Clb2-Cdk1. FHy336 (*clb6*Δ), FHy337 (*clb6*Δ *swe1*Δ), FHy338 (*clb5*Δ *clb6*Δ), FHy339 (*clb5*Δ *clb6*Δ *swe1*Δ), FHy340 (*clb5*::*CLB2 clb6*Δ), and FHy341 (*clb5*::*CLB2 clb6*Δ *swe1*Δ) cells, which all express Sld2–9Myc, were blocked in G_1 with α-factor and released at 23°C. Every 12 minutes, cells were harvested and proteins were extracted by TCA precipitation and subjected to immunoblot analysis with anti-Myc (9E10) antibody. The arrow indicates phosphorylated Sld2.

and Clb2-Cdk1. Clb5-Cdk1 phosphorylated the majority of Sld2 present, whereas Clb2-Cdk1 phosphorylated only about half of the available Sld2 (Fig. 3). Furthermore, the mobility shift of Sld2 was greater when phosphorylated by Clb5 than by Clb2, suggesting that only a subset of Sld2 phosphorylation sites were targeted by Clb2. Nevertheless, Clb2-Cdk1 phosphorylation of Sld2 clearly is sufficient to drive S-phase entry and support a normal rate of S-phase progression (Fig. 1*A*). Hence, transcriptional control and Swe1 regulation are potent mechanisms that restrict Clb2 function in the early cell cycle.

Clb2 Can Perform All Essential Cell Cycle Functions. *clb3–6*Δ cells are inviable, suggesting that Clb1 and Clb2 cannot perform an essential function(s) of Clb3–6 (4). However, we have demonstrated that Clb2 is capable of performing Clb5 and Clb6 replication function(s). Thus, lethality of *clb3–6*Δ mutant cells may result from the delayed activity of Clb2 (under its normal promoter and Swe1 regulation) relative to Clb3–6, or because Clb2 is unable to perform an essential function(s) of Clb3–6 other than DNA synthesis. If Clb2 is able to perform all of the essential functions of Clb3–6, then early expression of Clb2 and/or elimination of Swe1 might restore the viability of *clb3–6*Δ cells.

To determine whether Clb2 can substitute for essential Clb3-Clb6 function(s), we first examined whether elimination of Swe1 allows native Clb1 and Clb2 expression to rescue the viability of *clb3–6*Δ cells. We dissected 100 tetrads of a diploid strain heterozygous for *clb3–6*Δ and *swe1*Δ but did not recover *clb3–6*Δ or *clb3–6*Δ *swe1*Δ cells, indicating that endogenous Clb1 and Clb2 expression, even without Swe1 inhibition, cannot replace essential Clb3–6 function(s) (Table 1). To determine whether early expression of Clb2, with or without Swe1, can rescue the essential function(s) of Clb3–6, we analyzed the viability of haploid segregants of a diploid heterozygous for *clb3*Δ, *clb4*Δ, *clb5*::*CLB2*, *clb6*Δ, and *swe1*Δ. Of 176 tetrads dissected, we did not recover *clb3*Δ *clb4*Δ *clb5*::*CLB2 clb6*Δ cells, indicating that early expression of Clb2 alone cannot replace essential Clb3–6 function(s) (Table 1). However, early expressed Clb2 did rescue the viability of *clb3–6*Δ *swe1*Δ cells; we isolated 13 viable *clb3*Δ *clb4*Δ *clb5*::*CLB2 clb6*Δ *swe1*Δ spores, which was similar to their expected proportion (Table 1). Clb1 was not required for rescue by early expressed Clb2, because dissection of a diploid additionally heterozygous for *clb1*Δ yielded 10 viable *clb1*Δ *clb3*Δ *clb4*Δ *clb5*::*CLB2 clb6*Δ *swe1*Δ spores, which was similar to their expected proportion (Table 1). The good viability of cells lacking all Clbs except Clb2 was further demonstrated by their

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Table 1. Clb2 can perform all essential cell cycle functions

Spore genotype	Dissected tetrads	Total viable spores	Viable *SWE1* spores (expected no.), *P*	Viable *swe1*Δ spores (expected no.), *P*
*clb5*Δ *clb3*Δ *clb4*Δ *clb6*Δ	100	273	0 (9) <0.0001	0 (9) <0.0001
clb5::*CLB2 clb3*Δ *clb4*Δ *clb6*Δ	176	550	0 (18) $<10^{-9}$	13 (18)
clb5::*CLB2 clb1*Δ *clb3*Δ *clb4*Δ *clb6*Δ	72	236	0 (8) <0.0005	10 (8)

Spores of heterozygous diploid strains FHy180 (*swe1*Δ/*SWE1 clb5*Δ/*CLB5 clb3*Δ/*CLB3 clb4*Δ/CLB4 clb6Δ/*CLB6*), FHy181 (*swe1*Δ/*SWE1 clb5*::*CLB2*/*CLB5 clb3*Δ/*CLB3 clb4*Δ/*CLB4 clb6*Δ/*CLB6*), and FHy333 (*swe1*Δ/*SWE1 clb5*::*CLB2*/*CLB5 clb1*Δ/*CLB1 clb3*Δ/*CLB3 clb4*Δ/*CLB4 clb6*Δ/*CLB6*) were dissected, germinated at 30°C, and genotyped. The number of viable spores of each genotype is indicated. The probability *P* that the indicated strain is viable, but was not isolated because of random chance, is shown.

growth on rich medium, which was similar to that of *clb5*Δ *clb6*Δ cells (Fig. 4). Thus, Clb2-Cdk1 is inherently able to perform all essential Clb-Cdk1 functions; however, transcriptional regulation and inhibition by Swe1 normally restrict its intrinsically broad target range.

Discussion

Swe1 and Transcription Differentially Regulate the Function of B-Type Cyclins. This study shows that transcriptional control of cyclin gene expression and inhibition of Clb–Cdk1 complexes by Swe1 define two independent mechanisms that differentially regulate the function of CDKs in the early cell cycle. Previous studies had reported that early expression of Clb2 or Clb4 did not efficiently stimulate S-phase entry, suggesting that one or more DNA replication factors were poor substrates for mitotic CDK (11, 12). However, our findings attribute the poor S-phase promoting activity of Clb2 and Clb4 observed in the previous studies primarily to inhibition by Swe1, rather than to an intrinsically poor ability to interact with replication substrates. Indeed, in the absence of Swe1, early expressed Clb2, Clb3, or Clb4 were each able to stimulate S-phase entry with only a slight delay compared with Clb5 (Fig. 1).

Our results argue that Clb2 is an effective activator of S-phase and able to phosphorylate all essential cell cycle substrates. However, the limited phosphorylation of Sld2 and the slight delay in S-phase entry compared with Clb5 suggest that Clb2 is somehow defective in its interaction with at least a subset of S-phase substrates. While this manuscript was in revision, Loog and Morgan reported that Clb5-Cdk1 exhibits greater specificity than Clb2-Cdk1 toward a subset of CDK substrates, including Sld2 (27). Although Clb5-Cdk1 exhibits lower activity than Clb2-Cdk1 toward the majority of CDK substrates, its interaction with certain substrates is enhanced through a "hydrophobic patch" motif on Clb5 and an RXL motif on the substrate (27). Although Clb2 appears to lack this specific targeting motif, higher intrinsic activity of Clb2-Cdk1 (unrestrained by Swe1) may compensate for its lower specificity.

The relatively minor difference in the ability of Clb5- and Clb2-Cdk1 to target replication substrates *in vivo* suggested by our study contrasts with the great differences in the rates of substrate phosphorylations (up to three orders of magnitude) measured by Loog and Morgan by using purified proteins and emphasizes the difficulty in relating *in vitro* kinetic differences with an actual biological consequence. For example, incomplete Sld2 phosphorylation is sufficient for S-phase entry (Fig. 3) (26). However, redundancy of phosphorylation sites and differences in the importance of specific sites are not evident in bulk phosphorylation measurements. On the other hand, the sufficiency for S-phase entry may fail to reflect an unrecognized function of complete phosphorylation of Sld2 or other substrate(s) (see below). It is also worth noting that the much more rapid phosphorylation of Clb5-specific substrates *in vitro* occurred under conditions where the amount of Clb5-Cdk1 greatly exceeded the amount of Clb2-Cdk1 present (27).

To compare the relative kinase activities of Clb2- and Clb5-Cdk1 under our experimental conditions, we measured *in vitro* phosphorylation of histone H1, which represents a nonspecific CDK substrate (Fig. 5, which is published as supporting information on the PNAS web site). As expected, Clb2-Cdk1 attained a higher activity in the absence of Swe1; however, Clb5-Cdk1 was unaffected. In contrast to the kinetics of Sld2 phosphorylation (Fig. 3), H1 phosphorylation was detected sooner in immunoprecipitates from early expressing Clb2 extracts than Clb5 extracts. Thus, Clb5-Cdk1 is better at phosphorylating Sld2 than Clb2-Cdk1, whereas Clb2-Cdk1 is better at phosphorylating histone H1, consistent with the results of Loog and Morgan. Comparison of H1 kinase levels with the timing of S phase entry strongly suggests that a higher level of Clb2-Cdk1 than Clb5-Cdk1 H1 kinase activity is required to stimulate S-phase entry. In particular, the level of Clb2-associated H1 kinase activity when *clb5*::*CLB2 clb6*Δ *swe1*Δ cells began S-phase (between 24 and 36 min) was greater than Clb5-associated H1 kinase when *clb6*Δ *swe1*Δ cells began S-phase (between 12 and 24 min) (Fig. 5; see Fig. 1 for time of S-phase entry). Thus, differences in substrate specificities are apparent *in vivo* (e.g.: the kinetics of Sld2 phosphorylation and the timing of S-phase entry); however, transcriptional control and specific inhibition of Clb2-Cdk1 by Swe1 have greater functional consequences.

Swe1 Exhibits Specificity Toward CDKs. We have found that Swe1 differentially inhibits the replication-promoting function of CDKs in relation to their normal expression timing, with no inhibition of

Fig. 4. Clb2 can perform all essential cell cycle functions. Strains OAy470 (WT), DGy221 (*clb6*Δ), DGy228 (*clb5*Δ *clb6*Δ), FHy191 (*clb3*Δ *clb4*Δ *clb6*Δ), FHy116 (*clb5*::*CLB2 clb6*Δ), FHy354 (*clb1*Δ *clb3*Δ *clb4*Δ *clb6*Δ), FHy132 (*swe1*Δ), FHy136 (*clb6*Δ *swe1*Δ), FHy133 (*clb5*Δ *clb6*Δ *swe1*Δ), FHy214 (*clb3*Δ *clb4*Δ *clb6*Δ *swe1*Δ), FHy134 (*clb5*::*CLB2 clb6*Δ *swe1*Δ), FHy350 (*clb1*Δ *clb3*Δ *clb4*Δ *clb6*Δ *swe1*Δ), FHy209 (*clb5*::*CLB2 clb3*Δ *clb4*Δ *clb6*Δ *swe1*Δ), and FHy345 (*clb5*::*CLB2 clb1*Δ *clb3*Δ *clb4*Δ *clb6*Δ *swe1*Δ) were streaked onto yeast extract-peptone-dextrose medium and imaged after 2 days of growth at 30°C.

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earliest-expressed Clb5- and Clb6-Cdk1, intermediate inhibition of Clb3- and Clb4-Cdk1, and the strong inhibition of Clb2-Cdk1 (Fig. 1). Analysis of H1 kinase levels also indicates that Swe1 inhibits Clb2-Cdk1 but not Clb5-Cdk1 (Fig. 5). Thus, Swe1 seems capable of reinforcing the stepwise expression of cyclin pairs by modulating the activation of the succeeding cyclin pair. This function may help optimize the temporal execution and coordination of cell cycle events, particularly in relation to bud morphogenesis. In considering the insensitivity of Clb5 to Swe1, it seems important that Clb5 has relatively weak activity toward most substrates, because a broadly potent, Swe1-insensitive CDK would likely override the Swe1 checkpoint, even in the presence of a Swe1-regulated CDK such as Clb2-Cdk1.

The relative Swe1 sensitivity of the Clbs also correlates with their similarities to each other, with Clb5 and Clb6 being most divergent from Clb1 and Clb2 and with Clb3 and Clb4 lying in between them (28). Interestingly, each of these cyclin pairs differs from the others in the "hydrophobic patch" motif; however, a role for this motif in targeting cyclins other than Clb5 remains to be demonstrated. Further studies will be required to identify the exact features that distinguish the functions of Clb2, Clb3, and Clb4. It will also be quite interesting to identify the features responsible for their distinct susceptibilities to Swe1 inhibition.

Evolution of Distinct CDK Functions and the Importance of Checkpoints. It has been suggested that the relative timing of S- and M-phases in a primordial eukaryote could have been ensured by oscillation of the activity of a single CDK (i.e., without a need for cyclin-specific functions) if replication-promoting functions simply required a lower overall level of CDK activity than mitotic functions (2, 29, 30). The finding that Clb2-Cdk1 alone can sustain all cell cycle functions clearly supports this possibility. Similarly, in *Schizosaccharomyces pombe*, Cdc13 (which is functionally homologous to Clb2 and regulated by Wee1) can support cellular viability in the absence of the S-phase cyclin Cig2 (30). On the other hand, the relatively high level of Clb2-Cdk1 required to drive S phase (Fig. 5) argues against the premise that replication substrates might generally require a lower level of CDK function for activation to ensure that replication precedes mitosis. This finding suggests that without a highly specific mechanism for targeting replication substrates, the oscillation of a single CDK might not provide sufficient differentiation between the relative timing of S- and M-phases. Although CDK activity provides the driving force for execution of cell cycle events, much of the coordination between cell cycle processes is regulated by checkpoint kinases.

The evolution of cell cycle regulation by oscillation of a single CDK might have required the prior existence of cell cycle checkpoints to ensure that genome duplication be completed before its segregation (31, 32). Early expression of Clb2 and deletion of Swe1 did not notably advance cell division (data not shown), consistent with activation of a DNA and/or spindle checkpoint preventing premature mitotic entry that might result from early activation of a Clb2 substrate. In fact, we have detected activation of the DNA damage and replication checkpoint kinase Rad53 in the *clb5*::*CLB2 clb6*Δ *swe1*Δ cells (data not shown), consistent with the idea that a checkpoint acts to maintain the proper order of chromosome replication and segregation in cells lacking more specialized mechanisms of CDK regulation (including normal Clb2 transcription, Swe1 inhibition, and the presence of Clb5 and Clb6). An alternative, but nonexclusive explanation for activation of Rad53 in *clb5*::*CLB2 clb6*Δ *swe1*Δ cells is that Clb2 may be defective in phosphorylation of a Clb5- or Clb6-specific substrate or a specific phosphorylation site(s), which is not essential for S-phase but required to avert DNA damage during replication. This possibility may be exemplified by Sld2, which appears to be incompletely phosphorylated by Clb2-Cdk1 (Fig. 3).

In addition to regulation of CDKs, the temporal presentation of substrates (such as prereplicative complexes, replisomes, and kinetochores) probably also is a major factor in determining which CDK–substrate interactions occur. Cellular localization also plays a role in restricting a specific cyclin-Cdk activity. For example, *Xenopus* cyclin B-Cdk1, which localizes to the cytoplasm during S-phase, was recently shown to be capable of activating replication origins when relocalized to the nucleus (33). Together with our results, these findings and others discussed above suggest that the evolutionary diversification of B-type cyclins involves multiple mechanisms, including: (*i*) the emergence of new protein–protein interactions with a cyclin- or Cdk-specific regulator (such as Swe1), which does not necessarily require alteration of existing enzyme active site–substrate interactions, (*ii*) changes in expression timing, (*iii*) regulation of subcellular protein localization, and (*iv*) the emergence of specific cyclin–substrate interactions. Duplicated gene families have been indispensable participants in the evolution of protein diversity and, hence, multicellular organisms. This study has provided insights into the functional specialization of a critically important protein family.

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