Control of the yeast cell cycle by the Cdc28 protein kinase
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It is becoming increasingly apparent that the diverse functions of Cdc28 during the yeast cell cycle are performed by forms of the kinase that are distinguished by their cyclin subunits. Entry into the cell cycle at START involves the Cln cyclins. S phase needs Clb5 or Clb6 B-type cyclins. Bipolar mitotic spindle formation involves Clb1–4 B-type cyclins. Much of the order and timing of the cell cycle events may involve the progressive activation of Cdc28 kinase activities associated with different cyclins, whose periodicity during the cycle is determined by both transcriptional and post-transcriptional controls.

Introduction

The notion that a master regulator might orchestrate cell cycle progression stems primarily from cell fusion studies showing that the cytoplasm of M phase cells can cause nuclei from other stages to enter mitosis prematurely [1]. Biochemical and genetic searches for this regulator converged about 4 years ago with the discovery that an activity called maturation-promoting factor capable of causing frog extracts to enter M phase [2] was composed of a 34 kDa kinase. The homologues of this kinase, Cdc28 and cdc2, from two distant yeasts (Saccharomyces cerevisiae and Schizosaccharomyces pombe, respectively) had previously been shown through genetic analysis [3,4] to have key roles in regulating cell division. It has since become clear that many, if not all, eukaryotic cells contain multiple forms of this kinase, some concerned with commitment to the cell cycle, others with S phase, and yet others with various aspects of mitosis. It is now thought that the sequential appearance of these different forms could determine the order of many key cell cycle events. This review discusses our current understanding of the functions and regulation of the Cdc28 kinase in the budding yeast S. cerevisiae, for it is here that the notion of multiple kinase forms was born.

The Saccharomyces cerevisiae cell cycle

S. cerevisiae cells divide by budding to produce progeny that differ in several respects. The mother cell is usually larger than the daughter cell derived from the bud because the time between emergence of the bud and its abscission from the mother cell is less than the mass-doubling time [5]. It has been suggested that the asymmetrical mode of division of S. cerevisiae is atypical and this could cause its cell cycle controls to be exceptional [6]. In fact, many if not most, eukaryotic cells produce non-identical progeny. This property is just particularly obvious in budding yeast.

The fate of newly born G1 cells depends on their environment, their life cycle stage and their history. In the absence of nutrients, cells remain in G1 and enter a quiescent state, during which protein synthesis is much reduced, cells have stores of glycogen, and acquire tolerance to heat shock [7]. The failure to enter a new cell cycle and the acquisition of heat tolerance are probably independent responses to the lack of nutrients because slow growing G2 cells acquire similar levels of thermotolerance as slowly growing or stationary phase G1 cells; that is, arrest in G1 is not necessary for entry into a stress-resistant state (B Futcher, personal communication). In the presence of nutrients, G1 cells enter S phase only after a period of growth, whose duration depends on cell size at birth and is longer in daughter than in mother cells [5].

During the early G1 growth phase, haploid cells remain capable of conjugation, which is initiated by pheromones secreted by cells of opposite mating type. The mating pheromones not only induce genes involved in conjugation but also prevent cells entering S phase, with the result that mating partners arrest each other in G1 [8]. Shortly before S phase, haploid cells become refractory to cell cycle arrest caused by pheromone and no longer require nutrients for completion of the cell cycle. This point, which is called START, divides G1 into an early phase during which cells keep open several developmental fates, and a late phase in which cells are committed to completing the mitotic cell cycle [9]. The concept of

Abbreviations

SBF—Swi4/6-binding factor; SCB—Swi4/6 cell cycle boxes; MBF—Mlu-binding factor; MCB—Mlu cell cycle boxes.
START can also be applied to diploid cells, where it is defined as the point in G1 before which cells can begin meiosis and after which cells are committed to a further mitotic division. Size homeostasis is maintained by the requirement that cells achieve a minimum size before they can undergo START.

Preparations for all aspects of the cell division process occur soon after cells undergo START (Fig. 1): cells enter S phase, they duplicate their spindle pole bodies, the first step to forming a mitotic spindle [10], and lay down proteins required for cytokinesis in the vicinity of the future bud site [11]. The behaviour of mutants shows that these three events are independent of each other. The cdc4, cdc7, cdc34 and cdc53 mutants cannot enter S phase but they bud and duplicate their spindle pole bodies normally [9,12], whereas cdc31 and cdc24 mutants are specifically defective in spindle pole duplication [13] and localization of proteins to the prospective bud site [14], respectively.

Defining entry into G2 and M phases in S. cerevisiae has been a matter of some debate. It has been argued that S, G2 and M phases overlap [6]. The early duplication of spindle pole bodies may be widespread in fungi [15] and is analogous to the duplication of the centrioles during S phase. It is therefore not a suitable criterion for determining whether a cell has entered M phase. Duplicated spindle pole bodies remain closely attached to each other for a large fraction (> 25%) of the cell cycle and their separation to opposite poles of the nucleus and the formation bi-polar spindles does not occur until well after the completion of S phase, though significantly before nuclear division [10]. Thus, discrete S, G2 and M phases clearly exist. What is less clear is whether the appearance of a nuclear spindle apparatus should be considered the beginning of M phase. Until recently, there has been no other criterion because the nuclear membrane does not break down (as in all fungi) and chromosome condensation could not be detected. However, recent observations using in situ hybridization (FISH) suggest that ribosomal DNA is condensed approximately threefold in cells treated with nocodazole compared to mutants that fail to complete S phase but nevertheless form bipolar spindles (V Guacci, E Hogan, D Koshland, personal communication). M phase might therefore be best considered to begin only when cells have formed bipolar spindles and condensed their chromosomes. Cells that have formed bipolar spindles but have not yet condensed their chromosomes could be considered to be in a prophase-like state. In contrast, anaphase is relatively easy to detect. After certain gyrations in the neck between mother cell and bud [16], the DNA mass can be seen splitting into two

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**Fig. 1.** The abundance of cyclin transcripts and their functions during the cell cycle of S. cerevisiae. SPBD, spindle pole body duplication; SPBS, spindle pole body separation (i.e. the formation of bipolar spindles).
Cell multiplication

as the spindle pole bodies move further apart and the
nucleus becomes extended, a movement that would cor-
respond to anaphase B. Whether anaphase A precedes
this event is still unclear but this issue should be resol-
vable using FISH.

It is difficult to establish the phenotype of mutant cells
without knowing their precise history. Cell cycle stud-
ies are therefore best performed using synchronous cul-
tures. Three methods are particularly good for inducing
synchrony: release from G1 arrest induced by pheromone
[17]; release from a late anaphase block imposed by a
temperature-sensitive cdc15 mutation [18]; and the ma-
ipulation of G1 cyclin levels by their expression from the
GALI-10 promoter [19**]. All these methods are,
however, prone to artefacts and results are best con-
firmed using cultures obtained by centrifugal elutriation.
Pure populations of G1 daughter cells can be obtained by
elutriation as long as the cells are grown using subopti-
cmal carbon sources (such as raffinose). Such cultures are
particularly useful for comparing early and late G1 events,
but only a limited number of cells can be obtained.

The Cdc28 protein kinase

Fortune favoured yeast geneticists in their discovery of
Cdc28. Of the forty or so cell cycle genes identified on the
basis of mutant cells arresting uniformly at particular cell
cycle stages, cdc28 mutants were considered exceptional
because they were the only ones defective in START de-
spite normal rates of protein and RNA synthesis [20]. It is
now recognized that CDC28 has multiple roles during the
cell cycle [21,22,25**], and there is still no good expla-
nation why most alleles arrest uniformly in G1, mutants
that arrested throughout the cell cycle would have been
ignored! Furthermore, other genes required for START
have since been discovered (e.g. CDC27 and SIT4). If they
had been found at the same time as CDC28, there
would have been little reason to concentrate on the lat-
	er. Better evidence for an important regulatory role had
meanwhile emerged for the cdc2 gene from the distantly
related fission yeast S. pombe, in which there were alleles
that advanced the timing of mitosis [24]. The discovery
that cdc2 and CDC28 can complement each other and
that they encode homologous protein kinases was there-
fore of fundamental importance in bringing together two
different schools of cell cycle genetics [25–27].

Cyclins

Insight into how the Cdc2/Cdc28 protein kinase could
be involved in such diverse functions as DNA replica-
tion and mitosis had to await the discovery that its animal
homologue was an essential component of the previously
known growth-related histone H1 kinase and of an activ-
ity (known as maturation-promoting factor) from Xeno-
pus eggs capable of inducing in vitro metaphase arrest
(reviewed in [28]). Cdc2, Cdc28 and their animal coun-
terparts are only active as kinases when complexed with
a class of proteins called cyclins, which were initially
discovered by virtue of oscillations in their abundance
during sea urchin cleavage divisions [29]. Studies with
Xenopus extracts suggested that entry into metaphase
was triggered by the appearance of kinase activity asso-
ociated with B-type cyclins and that its destruction by
 proteolysis of the unstable cyclin subunit might initiate
anaphase [30].

A very different type of cyclin, now called Cln3, was soon
afterwards discovered in S. cerevisiae. Mutations that sta-
bilize Cln3 allow cells to undergo START at an abnormally
small size [31] or in the presence of pheromone [32].
This led to the hypothesis that the G1 and G2 functions
of Cdc2/Cdc28 are executed by forms of the kinase that
differ according to the type of cyclin subunit associated
with the kinase subunit; Cln and B-type cyclin associated
kinases were postulated to be active at START and mi-
tosis, respectively. At the time, this hypothesis suffered
from two major flaws: first, unlike mutations in CDC28,
deletion of CLN3 merely delays START; second, no B-type
cyclins had been detected in S. cerevisiae. Indeed, it was
argued that mitosis in S. cerevisiae was abnormal in that
it was initiated at START and might therefore use only
abnormal (Cln) cyclins.

Both problems have since been solved by the discovery
of many more cyclin genes in S. cerevisiae (Fig. 2). One
family, composed of CLN1, CLN2 and CLN3 is essential
for START [33], another, composed of B-type cyclins
encoded by CLB1, CLB2, CLB3 and CLB4, is necessary
for the formation and function of the mitotic apparatus
[23**],[34**],[35**]. CLB5 and CLB6, which encode an-
other pair of B-type cyclins, are important for S phase
(E Schwob, personal communication) [36**] and two
distant members of the family expressed in G1, HCS26
[37**] and ORFD (open reading frame D) [38], have
no known function. The rest of this review will con-
ider whether these cyclins are indeed activators of the
Cdc28 kinase, whether the kinase activity associated with
them is cell cycle dependent and if so how it is regulated,
and finally what are the consequences for a yeast cell of
changes in particular forms of Cdc28 kinase activity.

G1 cyclins and START

CLN1 and CLN2 were isolated due to their rescue of
a cdc28 mutant when overexpressed [39]. They en-
clude closely related proteins (58% identity) that are
only distantly related to Cln3 (23% identity). All three
Cln proteins are very different from the B-type cyclin
family involved in the control of mitosis (only 16–21%
identity). None of the three CLN genes are essential
for cell division. Furthermore, all double mutants are viable,
although their cell size is greatly increased. However,
deletion of all three genes is lethal and causes cells to
arrest as unbudded G1 cells [33] capable of conjugation
[40]. At least one of the three CLN genes is therefore
necessary for START.
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Fig. 2. A family tree of (complete) cyclin protein sequences from S. cerevisiae and S. pombe. The tree was constructed using the Wisconsin PILEUP program. *S. pombe cyclins.

Immunoprecipitates of each Cln protein contain a CDC28-dependent histone H1 kinase activity, which is much stronger for Cln1 and Cln2 than for Cln3 [41,42,43]; this difference may be significant given their very different protein sequences. These data do not yet prove that the essential function of Cln proteins is to activate Cdc28, although they are certainly consistent with the notion. The Cdc28 kinase activity associated with Cln1 and Cln2 is tightly cell cycle regulated [43]; it is absent in cells arrested in G1 by pheromone, but reappears soon after release, peaks in abundance as cells undergo START and declines as cells enter G2. The abundance of Cln1 and Cln2 proteins and transcripts has a similar pattern [41], which might therefore be determined by transcriptional control. In contrast, there is little or no cell cycle variation in the level of Cln3 transcripts, protein, or associated Cdc28 kinase activity [43]. Expression of CLN2 from the GAL1-10 promoter reduces the length of G1 (E Schwob, personal communication), suggesting that the onset of CLN1 and CLN2 transcription (as seen in wild type cells) may trigger START.

Regulators of CLN1 and CLN2 transcription

Two transcription factors called Swi4/6-binding factor (SBF) and MluI-binding factor (MBF; DSC1) have been implicated in late G1-specific transcription (reviewed in [44]). They have a common regulatory subunit encoded by SWI6 [45,46,48,49] but contain different site-specific DNA-binding subunits: Swi4 for SBF [49,50] and a 120kDa protein whose gene has not yet been characterized for MBF [46,48]. The known binding sites for SBF and MBF, swi4/6 cell cycle boxes (SCBs) and MluI cell cycle boxes (MCBs), respectively, look different, but both proteins can bind at least weakly to each other’s site [46,48]. Both types of binding site are sufficient to confer late G1-specific transcription on reporter genes and in some cases have been shown to be necessary for the transcription of genes whose promoters contain them [51,52]. Current evidence suggests that SBF, at least, has an important role in activating CLN1 and CLN2. Transcripts from both genes are nearly 10-fold less abundant in swi4 mutants [37,53,54], which prevents either gene from fulfilling its function. A role for MBF cannot yet be excluded because there are no mutants defective only for this factor; swi6 mutants are defective for both [46,47,55]. In certain genetic backgrounds, the lack of SBF function causes a lethality that is resuable by CLN2 expression from a foreign promoter [53] or by increased CLN2 gene dosage [37,53]. Mutants lacking SBF can also be rescued by increased gene dosage of the HCS26 gene [37], which encodes yet another type of cyclin (with approximately 25% identity to Cln and B-type cyclins). Like CLN1 and CLN2, HCS26 expression occurs as cells undergo START (A Amon, personal communication) and depends on SBF, but it is not yet known whether it is an activator of Cdc28 or whether it has a role at START. ORFD [38] encodes a similar type of cyclin (T Hunt, personal communication) that may be similarly regulated [43].

A positive feedback loop

CLN1, CLN2 and HCS26 transcripts are absent throughout the early G1 growth phase of daughter cells, but appear suddenly as cells undergo START (E Schwob, personal communication) [18,56]. Insight into the abruptness of their activation came from the discovery that it depends on an active Cdc28 kinase [19,56], suggesting that a positive feedback loop may be involved. It has been proposed that Cln/Cdc28 kinase activates SBF, which in turn activates CLN1/2 transcription, thereby closing the loop by further activating Cdc28 [37,53]. As predicted by this hypothesis, CLN1 and CLN2 activation is stimulated by G1 cyclin activity [19,56]. However, the importance of the positive feedback loop in the activa-
Cell multiplication

It is still not known how SBF is activated by Cdc28. A potential mechanism is the activation of SWI4 transcription, which is also maximal in late G1 [57]. However, SBF appears to be quite stable [48] and transcriptional activation of SWI4 is unlikely to cause more than a twofold increase in its concentration. Furthermore, genes regulated by SBF can be at least partly activated by Cdc28 in the absence of protein synthesis [58]. It is therefore likely that Cdc28 causes a post-translational modification of SBF. There are a change in the electrophoretic mobility of SBF–DNA complexes as cells undergo START, but it is not known whether this is due to the arrival a new protein (e.g. a cyclin) or due to phosphorylation of Swi4 or Swi6 by Cdc28 [48].

A positive feedback loop may help explain the apparent irreversibility of START. It is to be expected that the SBF/G1 cyclin regulatory circuit would have only two stable states: one with low kinase and a second with high kinase. The transition from the low to the high kinase state may be the biochemical basis to START. Repression of CLN1 and CLN2 as cells enter G2 depends on the mitotic cyclins Cib1–4 (A Amon, personal communication).

Other genes required for START

Mutations in many genes cause yeast cells to arrest in G1. In most cases, however, arrest can be explained as a secondary effect due to defective protein synthesis. For example, protein synthesis is greatly reduced in cdc28 and cdc35 mutants, which are defective in cAMP production (reviewed in ref [7]), whereas transcription from many POL1 genes is defective in cdc68 mutants [59]. There are in fact surprisingly few mutants whose G1 arrest resembles that of cdc28 or triple cln mutants, which continue to grow rapidly in the absence of cell division. Of these, the best characterized are cdc37 [60] and sit4 mutants [61]. The function of Cdc37 is unknown and SIT4 encodes a phosphatase. Loss of SIT4 function is only lethal in cells mutated in a second gene called SSD1 (or SRK1), which is homologous to the dis3 gene in S. pombe and could encode a phosphatase regulatory subunit. The sit4 red1 double mutants normally arrest as unbudded cells in G1, but expression of CLN2 from a foreign promoter allows them to undergo S phase, although they still cannot bud [62]. This suggests that Sit4 may have a role not only in activating G1 cyclins (possibly via Swi4) but also in bud formation. It is interesting that both phosphatases and kinases are needed for START. Might the Sit4 phosphatase be required to prepare substrates for activation by the Cdc28 kinase?

Size control

START only occurs when cells reach a critical cell size [9]. Such a property ensures the coordination between cell division and growth, but how it is achieved is not yet understood. A good guess is that there are mechanisms for cyclin activation that are not dependent on the pre-existence of kinase activity and are somehow proportional to cell size. The Cln3 protein, which does not oscillate much during the cell cycle, is ideally suited for this function [43]. Deletion of CLN3 is not lethal but causes cells to delay START until they are twice their normal size [31,32]. There may therefore exist more than one mechanism for triggering START. A Cdc28-independent pathway for activating CLN2 transcription is another possibility [63].

It is worth considering how size control might function. Reducing protein synthesis with cyclohexamide delays START, suggesting that the critical parameter for initiation is size per se but achieving a critical rate of protein synthesis (reviewed in [64]). G1 cyclins are unstable proteins and their amount per cell will therefore reflect their rate of synthesis, which, like most other proteins, will be proportional to cell size. Thus, if Cln3 or proteins like it are accumulated in the nucleus, then their concentration (or amount relative to chromatin) will increase as cells grow. The dynamics of a positive feedback loop are such that it will be triggered from the low to high kinase state as soon as a critical level of an activator like Cln3 is reached, i.e. very small changes in the rate of Cln3 synthesis could trigger START. As predicted by this model, increased Cln3 expression from the GAL1–10 promoter can trigger START prematurely in small daughter cells [43].

Nutrient control

There are two aspects to the control of START by nutrients. The first concerns the failure of cells to undergo START when starved [9]. So far, there is no evidence that G1 cyclins or Cdc28 are directly affected by nutrient status, though it is entirely possible. However, there may be no need to invoke a special mechanism. G1 arrest caused by starvation could be a simple consequence of lowered rates of protein synthesis (as discussed in [9]). The second aspect is of more obvious interest and concerns the control of cell size by nutrients. In general, cells growing in media that support fast rates of growth are larger than cells growing in poor media, an effect that is due to differences in the minimum size required for START [65]. It seems that fast-growing cells find it harder to undergo START than slow-growing cells, which is all the more striking when it is considered that the latter have lower rates of protein synthesis. A similar phenomenon has been reproduced in certain yeast strains by adding cAMP to the medium [66]. In this case, the cell size required for START is increased by elevating cAMP levels,
i.e. cAMP acts as an inhibitor of START (which contrasts with the earlier notion that cAMP is an activator). In this context, it is striking that the levels of \(CLN1\) and \(CLN2\) transcripts are high in \(cdc25\) mutant cells arrested in \(G_1\) [62**]. These cells have low rates of protein synthesis and presumably do not undergo START because \(G_1\) cyclins are not synthesized and it is frankly surprising, in the light of the positive feedback model, that they transcribe \(CLN1\) and \(CLN2\). The phenomenon could be explained if down regulation of \(CLN1\) and \(CLN2\) transcription by \(Cdc25\) (via cAMP) was part of the mechanism by which nutrients controlled the cell size needed for START. The small size of \(cdc25\) mutant cells growing with a wild type generation time at the permissive temperature is consistent with this notion [67].

Is an oscillation needed for START?

The notion that a wave of Cdc28 activity might be responsible for START has been entertained ever since \(CDC28\) was found to encode a protein kinase. However, despite progress in understanding the dynamics of \(CLN1\) and \(CLN2\) regulation, it is still not clear whether an oscillation is essential for START. In \(cln1\) \(cln2\) double mutants, \(CLN3\) is now essential, but this cyclin does not appear to be regulated. Furthermore, cells lacking all three \(G_1\) cyclins can be kept alive by expression of \(CLN1\) or \(CLN2\) from constitutive promoters [33,53**]. The quality of life for such cells may be poor, but their survival in the laboratory suggests that a steady increase in cyclin activity could also accomplish START. This issue will not be resolved until we know more about post-translational controls on cyclin activity and more about other genes contributing to START whose activity is regulated. For example, evidence that Cln3 is activated by phosphorylation raises the possibility of a positive feedback loop involving auto-phosphorylation [42**]. Furthermore, cells lacking the regulated \(CLN1\) and \(CLN2\) genes still contain \(HC26\), \(CLB5\) and \(CLB6\), which encode tightly cell cycle regulated cyclins possibly active at START (E Schwob, personal communication).

Regulation of START by pheromone

The activation of genes involved in conjugation (such as \(FUS1\)) and the inhibition of START are, by and large, independent responses of haploid cells to their mating pheromones. \(FUS1\) induction is not dependent on cell cycle arrest and there exist mutants that induce \(FUS1\) normally but nevertheless fail to undergo arrest (see below). Both responses rely on the same signal transduction pathway (reviewed in [8]). Pheromone binding to surface receptors releases the \(\beta\)-subunit of a G protein from an inhibitory \(\alpha\)-subunit, and this somehow activates a kinase cascade, through which the Fus3 kinase (which is homologous to mammalian MAP kinases) is activated by the Ste7 kinase [68]. Dominant alleles of \(CLN3\) that produce a more stable protein [32,42**] or recessive mutations that inactivate the \(FAR1\) gene [69] allow cells to continue division despite inducing genes like \(FUS1\) normally. The pheromone resistance of \(far1\) mutants depends on a functional \(CLN2\) gene. Pheromone-induced \(G_1\) arrest is therefore thought to involve the inhibition of \(G_1\) cyclin activity and the inhibition of \(CLN2\) at least depends on \(FAR1\). \(CLN1\) and \(CLN2\) transcripts decline when cells are treated with pheromone [41], but low-level expression of \(CLN2\) from an unregulated promoter does not cause pheromone resistance (G Ammerer, personal communication) [70]. Thus, the inhibition of \(CLN2\) activity (by \(FAR1\)) must occur at a post-transcriptional level. Indeed, inhibition of Cdc28 may be due to a pheromone-induced association of the Far1 protein with Cln1 and Cln2 (M Tyers, B Futter, and M Peter, A Gartner, J Horecker, G Ammerer, I Herskowitz, personal communications). Repression of \(CLN1\) and \(CLN2\) transcription could be a secondary consequence of inhibiting \(G_1\) cyclin activity, as predicted by the positive feedback loop model. It could nevertheless contribute to the efficiency of arrest because high level expression of \(CLN2\) does cause pheromone resistance. \(FAR1\) transcripts are induced by pheromone, but this is not the sole mechanism by which Far1 activity is activated, because \(FAR1\) expression from the \(GAL\) promoter does not cause \(G_1\) arrest. It is possible that Far1 is activated due to phosphorylation by the Fus3 kinase [68]. How Cln3 stabilization causes pheromone resistance is still unclear because overproducing the wild type protein from the \(GAL\) promoter does not generate the same phenotype [42**].

Consequences of \(G_1\) cyclin activity

Little is known about physiological substrates for Cln/Cdc28 kinases. S phase entry occurs soon after the appearance of \(CLN1/2\) transcripts and requires the \(CDC4\), \(CDC7\), \(CDC34\) and \(CDC53\) genes, none of which are required for START in haploids [9] or for the activation of \(G_1\) cyclins. \(CDC34\) encodes a ubiquitin conjugating enzyme, which has led to the proposal that degradation of \(G_1\) cyclins might be necessary for the transition from START into S phase, just as the degradation of \(G_2\) cyclins is necessary for exit from mitosis [71]. Cells arrested by \(cdc34\) do contain high levels of \(CLN1\) and \(CLN2\) transcripts (A Ammon, personal communication) and have greatly elevated Cdc28-dependent kinase activity associated with Cln3, which may be due to the accumulation of a hyper-phosphorylated form [42**]. However, several facts seem at present inconsistent with the notion that DNA replication is hindered by high \(G_1\) cyclin activity: high level constitutive \(CLN2\) expression functions to accelerate rather than delay S phase (E Schwob, personal communication); \(cdc34\) arrest does not depend on \(CLN3\) [42**]; and S phase occurs normally in other mutants in which \(Cln1\) and \(Cln2/Cdc28\) kinases fail to be repressed (A Ammon, personal communication). \(CDC4\) encodes a protein homologous to \(\beta\)-transducins [72].
and CDC7 encodes a protein kinase [73], but little is known about their function or whether they might be substrates for Cdc28. A complex that binds origins has recently been isolated [74**]. One or more of its constituents could be a substrate for Cdc28.

Many genes involved in DNA replication are activated transiently as cells undergo START (reviewed in [75]). Their transcription does not require SWI4 but is de-regulated in swi6 mutants and depends on MCBs, implicating MBF (and not SBF) in their regulation [46**.47**]. Gene activation by MBF requires the Cln G1 cyclins and Cdc28. Most of the genes regulated by MBF encode stable proteins whose inheritance from the parental cell is sufficient for S phase. Nevertheless, MBF probably also regulates CLB5 and CLB6, which encode B-type cyclins involved in S phase entry (E Schwob, personal communication). Thus, MBF activation may play an important part in the initiation of DNA replication.

It is not known how the activation of a Cln/Cdc28 kinase leads to the duplication of spindle pole bodies (Fig. 1) or to bud emergence. Neither of these events require the known G2 cyclin genes CLB1, CLB2, CLB3 and CLB4 [31**], and they could therefore be under direct control of G1 cyclins. Over-expression of stable G2 cyclins not only causes mitotic arrest but also inhibits bud formation [76**], a phenomenon that could be explained if G1 cyclins have a direct role in bud formation, which is hindered by an excess of G2 cyclins.

### G2 cyclins and mitosis

*S. cerevisiae* contains at least four genes (CLB1-4) encoding B-type cyclins that are involved in mitosis. They comprise two families: CLB1/CLB2, which are 62% identical, and CLB3/CLB4, which are 50% identical (Fig. 2). The Clb1/2 pair is the more closely related to B-type cyclins from animals. Of the four genes, CLB2 seems to have the most important role in mitosis. Deleting CLB1, CLB3 and CLB4 has little or no effect on cell division [34**,35**], whereas deletion of CLB2 alone greatly delays the onset of mitosis [23**]. The phenotype of cells lacking activity of all four genes has been analyzed using strains whose CLB1, CLB3 and CLB4 genes are deleted and whose CLB2 gene is conditionally active due to a temperature-sensitive mutation (A Amon, personal communication) or to expression from the GAL promoter (which can be repressed) [34**]. Upon inactivation of CLB2, cells arrest in G2 with duplicated spindle pole bodies and large buds. The duplicated spindle pole bodies remain attached, as they do in cdc4 mutants, and consequently no bipolar spindle is formed. G1 cells were seen to initiate and apparently complete S phase in the absence of CLB1, CLB2, CLB3 and CLB4. In a separate study, in which a quadruple clb mutant was kept alive by expression of CLB1 from the GAL promoter [35**], cells also seemed to have difficulties in completing S phase when CLB1 was repressed. Whereas CLB3 and CLB4 can be deleted with little or no effect on cell cycle progression, mutation of CLB1 and CLB2 is almost lethal and causes cells to arrest with bipolar spindles (A Amon, personal communication) [23**]. The CLB1/2 and CLB3/4 cyclin types therefore have different properties; either the CLB1/2 pair or the CLB3/4 pair can promote the separation of duplicated spindle pole bodies and the formation of a bipolar spindle, but the CLB1/2 pair is necessary for subsequent phases of mitosis, which could include chromosome condensation or aspects of kinetochore function. At the moment, it would seem as if CLB2 may be able to perform both early and late functions on its own. A caveat to this conclusion is that we do not know for sure whether CLB1-4 are the only B-type cyclin genes active in mitosis in yeast. There may well be other genes, without whose function CLB2 cannot alone drive cells through mitosis.

### Cell cycle regulation of G2 cyclins

Immunoprecipitates of Clb1, Clb2, Clb3 or Clb4 have CDC28-dependent histone H1 kinase activities that are tightly cell cycle regulated and seem much stronger than those found associated with G1 cyclins [43,77**,78]. Clb2-associated kinase is absent in cells arrested in G1 by pheromone. Its appearance upon release occurs after DNA replication (around the time that cells form a bipolar spindle) and precedes anaphase by at least 15 min [79]. Clb2 protein and kinase activity are destroyed as cells exit from mitosis, as is found for B-type cyclins in other eukaryotes. Clb1 kinase is similarly regulated, but Clb3 and Clb4 kinases appear somewhat earlier [78].

CLB1 and CLB2 transcripts have a similar profile to Clb2 kinase activity, appearing in G2 and disappearing as cells enter G1 [23**,76**]. CLB3 and CLB4 transcripts appear earlier (but still later than CLN1 and CLN2), which is consistent with their function in an earlier stage of mitosis [34**,35**]. How much transcriptional control contributes to the regulation of Clb2/Cdc28 kinase activity is not yet known because the consequences of premature activation or delayed repression have not yet been carefully analyzed. Little is known about the transcription factors responsible for regulating Clb2 transcription. CLB1 and CLB2 regulation appears very similar to that of the SWI7 gene, whose promoter requires the formation of a ternary complex containing Mcm1 and a factor called Swi five factor (SFF) [80]. Of these, SFF is the better candidate for a G2-specific transcription factor, because Mcm1 is also involved in the activation of genes that are not cell cycle regulated [81].

An important question is whether the RAD9-dependent inhibition of mitosis by DNA damage [82] (Fig. 1) is due to regulation of one or another form of the Cdc28 kinase. DNA damage arrested cells, e.g. cdc13 mutants, arrest with bipolar spindles, a phenotype that is similar to that of clb1 clb2 double mutants. However, Cdc28 kinase activity associated with Clb2 in arrested cdc13 mutants is at least 50% of that found in cells arrested with nocodazole [77**]. The possibility that mitosis can be regulated...
by means that do not directly involve Cdc28 must therefore be seriously considered.

Roles of Cdc28 phosphorylation and cyclin proteolysis

In several other eukaryotes, Cdc2/Cdc28 kinase activity associated with B-type cyclins is largely determined by post-transcriptional controls (reviewed in [28]). In S. pombe, Cdc2 kinase is inhibited during G2 by phosphorylation of Tyr15 by the Wee1 family of kinases. Dephosphorylation by the Cdc25 phosphatase seems to determine the onset of M phase. Mutation of Tyr15 or the wee1 gene causes premature entry into M phase. The equivalent residue in Cdc28 (Tyr19) is also phosphorylated in a cell cycle dependent manner [77,83]. Moreover, there exist homologues to the Wee1 kinase (B. Booher, personal communication) and the Cdc25 phosphatase (called MIH1 [84]). Despite this, mutation of Tyr19 to Phe has little or no effect on the length of G2 or the inhibition of nuclear division by unreplicated or damaged DNA [77,83]. It would seem that the stoichiometry of Cdc28 Tyr19 phosphorylation is not sufficient to affect Cdc28 kinase activity greatly, although it appears that the kinase can be inhibited and cells prevented from undergoing nuclear division if the stoichiometry of phosphorylation is increased by over-expressing S. pombe Wee1 kinase in mit1 mutants [84].

Tyr19 is phosphorylated in cells prevented from undergoing nuclear division due to unreplicated or damaged DNA (e.g. cdc13 mutants) but is unphosphorylated in cells arrested due to microtubule defects (e.g. in nocodazole) [77,83]. FISH analysis suggests that ribosomal DNA is less condensed in arrested cdc13 mutants than in nocodazole (V Guacci, E Hogan, D Koshland, personal communication). Thus both the analysis of Cdc28 phosphorylation and the state of ribosomal DNA condensation suggest that cdc13 arrest is G2-like and nocodazole arrest M phase-like. Because cdc13 mutants have bipolar spindles, they may be better considered as arresting in prophase.

The abrupt destruction of B-type cyclins during anaphase in Xenopus is conferred by a conserved sequence towards their amino termini, called a destruction box [30,85]. All four mitotic Cib cyclins in yeast contain sequences similar to the canonical destruction box, and mutation of the sequence in CIB2 causes stabilization [79]. It therefore seems likely that the destruction machinery is conserved between Xenopus and yeast. Cyclin variants that cannot be destroyed because of mutations in their destruction boxes are reported to cause Xenopus extracts to arrest in metaphase; and this has led to the proposal that destruction of B-type cyclins signals the metaphase to anaphase transition [30]. Expression of stable versions of Cib1 or Cib2 cause a lethal cell cycle arrest in yeast, but the arrest does not correspond to metaphase. Cells arrest with greatly extended bipolar spindles and DNA masses that seem to have completely segregated, indicating that anaphase B at least has occurred [79]. Another piece of evidence inconsistent with anaphase being triggered by cyclin destruction is the behaviour of cdc15 mutants, which arrest in a cell cycle state similar to that caused by stable cyclins. There is little or no drop in Cib2/Cdc28 kinase activity as cdc15 cells undergo anaphase [79]. The kinase only disappears when cells are allowed to exit from late anaphase/telophase upon return of the mutant cells to the permissive temperature. Thus, cyclin destruction in yeast (at least) seems required for the final exit from mitosis and not, as previously thought [76], for the initiation of anaphase.

Targets

Very little is known about physiological targets of mitotic forms of Cdc28. The only example so far is the Swi5 transcription factor, which accumulates in the cytoplasm during G2 and M phases and only enters the nucleus as cells enter G1 [86]. The nuclear localization signal of Swi5 is phosphorylated by Cdc28 and this prevents nuclear uptake [87]. Destruction of the kinase at the end of mitosis leads to dephosphorylation and entry into the nucleus. Swi5 is required for transcription of the HO endonuclease gene involved in mating type switching and does not have an essential cell cycle function [88]. However, a related transcription factor, called Ace2, may be regulated in a similar manner [89] and is required for the activation of a chitinase gene (CTSI) involved in cell separation as cells exit from mitosis (R Siegmund, personal communication). The activation of transcription factors through destruction of the Cdc28 kinase at the end of mitosis may be quite a general phenomenon. Several genes, in addition to CTSI, are only transcribed as cells enter G1 [18]. The regulation of these early G1 genes is distinct from those activated in late G1 as cells undergo START, in that Cdc28 seems to repress the former but activate the latter.

Conclusions and questions

The budding yeast S. cerevisiae has at least 11 different genes that encode cyclin-like proteins, many of which have been shown to be associated with an active Cdc28 kinase. Some genes are required for START, others for DNA replication, and others for various steps in the assembly and function of a mitotic spindle (Figs 1 and 2). If we compare the cell cycle to a clock, it would seem that changes in Cdc28 activity not only sound the passing of the cell cycle 'day' (i.e. the passing from one cell cycle to the next) but also register the hours within that day.

Much remains to be discovered about the extent to which the timing of cell cycle events is determined solely by the activation of different cyclins. Only in the case of G1 cyclins is there evidence that premature cyclin activation can actually advance cell cycle events. Furthermore, it is still quite unclear what distinguishes the activity of differ-
different types of cyclins. What property of G₁ cyclins enables them exclusively to activate transcription at the beginning of the cell cycle and what property enables G₂ cyclins to promote the formation of a bipolar spindle? To what extent is this due to different substrate specificities and to what extent is this due to them being active at the correct time and in the correct cellular compartment? Identifying important substrates for different forms of the kinase should be a high priority in future studies.

Major questions remain concerning the dynamics of cyclin oscillations. Our current picture is as follows. Cells that have just entered G₁ start off with possibly only Cln3 protein, whose amount increases as cells grow, at some critical cell size CLN1 and CLN2 transcription is partially activated. Cln1 and Cln2 proteins then reinforce Cln3, leading to more CLN1/CLN2 transcription and thereby to high levels of Cln/Cdc28 kinase activity. At some point during this process, the Cln/Cdc28 kinase activates transcription of other cyclin genes (like HCS26, CLB5 and CLB6) and a battery of genes involved in DNA replication. Somewhat later, CLB3 and CLB4 transcripts appear and, later still, those from CLB1 and CLB2. Meanwhile, transcription of CLN1, CLN2, HCS26, CLB5 and CLB6 declines. CLB1 and CLB2 transcripts and kinase activity remain until some event during anaphase causes B-type cyclins with destruction boxes to be degraded and their genes to be repressed. Little is known about post-transcriptional controls over Cdc28 kinase activity associated with either Cln or Clb proteins, what causes the decline of CLN and the rise of CLB transcription as cells enter G₂ and what causes the destruction of B-type cyclins, which is so important for exit from mitosis and for re-setting the cell cycle clock.

Another question of fundamental importance is the extent to which the successive waves of cyclin oscillations are dependent on each other rather than on the completion of events set in motion by earlier cyclins. For example, what role do G₁ cyclins have in activating G₂ cyclins and what role do they in turn have in repressing G₁ cyclins? Must events such as spindle pole body duplication and DNA replication be complete before G₂ cyclins can be activated? It would seem not in the case of the latter, because Cdc28 kinase associated with Clb2 becomes elevated in cdc7 mutants, which cannot initiate DNA replication. The only aspect of cyclin control that is clearly dependent on the completion of cell cycle events is the destruction of mitotic cyclins following nuclear division. Many mutants defective in DNA replication or mitosis do not proceed to destroy mitotic cyclins. Enzymes involved in DNA replication, for example, are unlikely to be directly involved in cyclin proteolysis, so the cell must somehow register the failure of DNA replication and communicate this (however indirectly) to the destruction machinery. Such mechanisms have been called checkpoint controls [90].

It is in fact surprising how many mutants totally defective in nuclear division proceed with the activation and destruction of mitotic cyclins as if nothing had gone wrong with mitosis (e.g. [91]). Indeed, it is now clear that the initial cdc mutants collected by Hartwell [3] were highly biased by the requirement (a vital one at the time) that cells arrest with a uniform morphology. Many, if not most, mutants with defects to do with cell division do not in fact arrest uniformly because the cells cannot register the defect and shut down the oscillations of cyclin/Cdc28 activity. It seems that yeast cells check only certain cell cycle events (which include DNA damage [82] and the integrity of microtubules [92**96**, presumably those that are most likely to go wrong in the natural world. In this regard, yeast may not be that different from early frog embryos whose cyclin/cdc2 oscillations do not even require a nucleus [94].

**Comparisons**

The notion that different cell cycle events are regulated by forms of the Cdc2/Cdc28 kinase distinguished by their cyclin subunits may prove to be a conserved feature of the eukaryotic cell cycle, although the details differ considerably. In vertebrate cells, there exist at least four classes of cyclins [95]: E-type cyclins, which appear in late G₁, may be analogous to Cln1 and Cln2, A-type cyclins, which appear at the beginning of S phase and seem important for its completion, may be analogous to Clb5; B-type cyclins, which appear in G₂ and disappear as cells undergo anaphase, are similar to Clb1 and Clb2; and D-type cyclins, which are less tightly cell cycle regulated, could in this regard be analogous to Cln3. Of these different classes, only the B-type cyclins are obviously homologous when their primary sequences are compared. For example, the sequences of G₁-specific cyclins in vertebrates and yeast are not more related to each other than either to B-type cyclins from either organism. Another difference between yeast and vertebrates is that the latter not only have multiple cyclin types but also multiple Cdc2/Cdc28 kinase subunits. Early cyclins seem to associate with the Cdk2 kinase subunit [96,97], whereas late cyclins associate with Cdc2.

In *S. pombe* as in *S. cerevisiae*, a single Cdc2 kinase subunit is required for both START and mitosis [98]. Three cyclin-like genes have been reported (Fig. 2): a B-type cyclin encoded by *cdc13*, which is required for mitosis [99]; a second B-type cyclin called Cig1, which is most closely related to Clb3 and Clb4 [100**]; and a D-type cyclin, which is less tightly cell cycle regulated in *S. pombe* as in *S. cerevisiae*, and is a component of the MBF/DSC1-like factor that binds to MCB elements [102**]. It seems that transcriptional activation plays an important part in both yeasts. What is still unclear is what genes are regulated by the *S. pombe* MBF/DSC1.
life cycle of S. pombe is such that it has a haploid vegetative phase [6], which may explain why it spends as little time as possible in G1, when haploid cells are particularly sensitive to DNA damage [63]. Both size and nutrient controls in S. pombe are therefore exerted in G2 and rely on control of Cdc2 phosphorylation, which is relatively unimportant for S. cerevisiae [77••,83••].

Certain conclusions can be drawn from comparing cell cycle control in these organisms: first, G1 cyclins are much less conserved than the B-type cyclins involved in mitosis; second, in fungi at least, B-type cyclins have very diverse functions; third, the machinery for cyclin destruction at the end of M phase may be highly conserved; and finally, the enzymology for controlling phosphorylation of Tyr15 in Cdc2/Cdc28 is also highly conserved, even though the uses to which it is put are not. The universal instability of cyclins presumably enables signals from outside or inside the cell to affect rapidly their concentration and thereby biological activity. One suspects that our distant ancestors used a B-type cyclin to beat out its cell cycle rhythm with periodic cyclin proteolysis being a fundamental aspect of the oscillator (i.e. the heart of the cell cycle). Cdc2/Cdc28 tyrosine phosphorylation may have served to distinguish S from M phase. It must be significant that the more ancient aspects of cell cycle control are used to regulate mitosis. Is this because the fundamental mechanisms that regulate DNA replication evolved before the evolution of eukaryotic organisms? If this is the case, cyclins and Cdc2/Cdc28 may have evolved originally to regulate mitosis and have only more recently been used to exert control over DNA replication and passage through G1. An alternative explanation is that the common ancestor of eukaryotes was a haploid organism, in which control of the cell cycle was exerted predominantly in G2.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as: • of special interest •• of outstanding interest


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Cell multiplication


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