

## REPORT

# Cell cycle regulation by feed-forward loops coupling transcription and phosphorylation

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**The eukaryotic cell cycle requires precise temporal coordination of the activities of hundreds of ‘executor’ proteins (EPs) involved in cell growth and division. Cyclin-dependent protein kinases (Cdks) play central roles in regulating the production, activation, inactivation and destruction of these EPs. From genome-scale data sets of budding yeast, we identify 126 EPs that are regulated by Cdk1 both through direct phosphorylation of the EP and through phosphorylation of the transcription factors that control expression of the EP, so that each of these EPs is regulated by a feed-forward loop (FFL) from Cdk1. By mathematical modelling, we show that such FFLs can activate EPs at different phases of the cell cycle depending on the effective signs (+ or –) of the regulatory steps of the FFL. We provide several case studies of EPs that are controlled by FFLs exactly as our models predict. The signal-transduction properties of FFLs allow one (or a few) Cdk signal(s) to drive a host of cell cycle responses in correct temporal sequence.**

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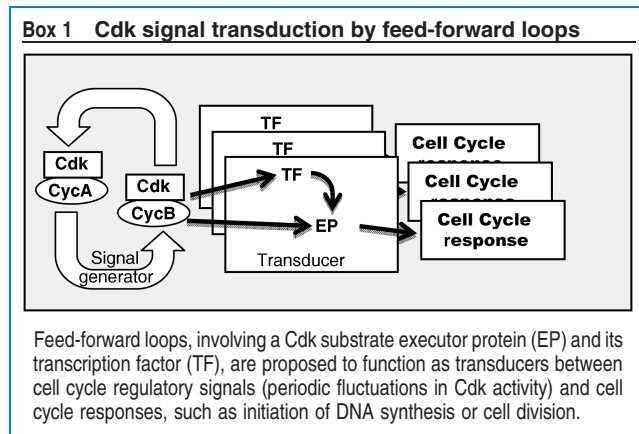
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## Introduction

A eukaryotic cell’s progression through G1, S, G2 and M phases of the cell replication division cycle is orchestrated by large-amplitude fluctuations in Cyclin-dependent protein kinase (Cdk) activities that are generated by a series of coupled positive and negative feedback loops (Novak *et al*, 2007; Holt *et al*, 2008; Skotheim *et al*, 2008; Tyson and Novak, 2008). Cdk signals are transduced into appropriate cell cycle responses by specific executor proteins (EPs) (Sutani *et al*, 1999; Tanaka *et al*, 2007a) (Box 1). For example, cell division is controlled by Cdk1 phosphorylation of components of a signalling pathway called the ‘mitotic exit network’ in budding yeast and the ‘septation initiation network’ in fission yeast (Bardin and Amon, 2001). Recently, we showed (Csikász-Nagy *et al*, 2007) that the

septation initiation network has the characteristic topology of a feed-forward loop (FFL): the high level of Cdk1–cyclin B in mitosis activates proteins that function early in the network (sensors) and inactivates proteins that function late in the network (executors). High Cdk1 activity primes the septation initiation network, but the network cannot ‘fire’ until Cdk1 activity falls and releases the inhibitory arm. A similar FFL controls the onset of DNA synthesis, according to the ‘licensing factor’ hypothesis (Blow, 1993). Recognizing the roles of FFLs in executing DNA synthesis and cell division, we hypothesized that FFLs might be common motifs in transmitting signals from Cdk1–cyclin master regulatory complexes to target proteins that execute cell cycle events.

Cdk1 substrates are potential EPs, as are proteins that are periodically expressed during the cell cycle (Spellman *et al*,



1998; Jensen *et al*, 2006). Intriguingly, proteins that are periodically expressed during the cell cycle are often Cdk substrates (Ubersax *et al*, 2003; Jensen *et al*, 2006). Furthermore, the transcription factors (TFs) that drive cell cycle-dependent gene expression must be cell cycle-regulated themselves, and it is reasonable to suspect that at least some of them are phosphorylated by Cdks. Wherever this is the case, the Cdk-TF-EP trio are involved in an FFL (Box 1). Owing to large-scale experimental screens in budding yeast (*Saccharomyces cerevisiae*) for targets of Cdk1 (Ubersax *et al*, 2003; Loog and Morgan, 2005), as well as for cell cycle TFs (Lee *et al*, 2002), it is possible to systematically test this hypothesis at the genome-wide scale.

## Results and discussion

To this end, we classified all the 4691 verified protein-coding genes of the budding yeast genome into 6 non-overlapping network topologies (Figure 1A) based on whether or not the encoded protein has been reported to be a Cdk1 substrate, whether or not TFs of the gene are known and whether or not at least one TF is a Cdk1 target. We identified 126 genes involved in an FFL, that is the encoded protein is a Cdk1 target and at least one TF is a Cdk1 target. Of these 126 genes involved in FFLs, 68 (54%) are found to be periodically expressed during the cell cycle, whereas only 13 would be expected by chance ( $P < 10^{-28}$ ). None of the other regulatory motifs shows a comparably high ratio of periodically expressed genes (Figure 1A; Supplementary Table S1). Thus, it is clear that a strong predictor of cell cycle periodicity is the involvement of a gene in an FFL regulatory motif. This observation suggests that the 68 periodically transcribed, FFL-regulated proteins (Supplementary Table S2) may indeed be key cell cycle EPs.

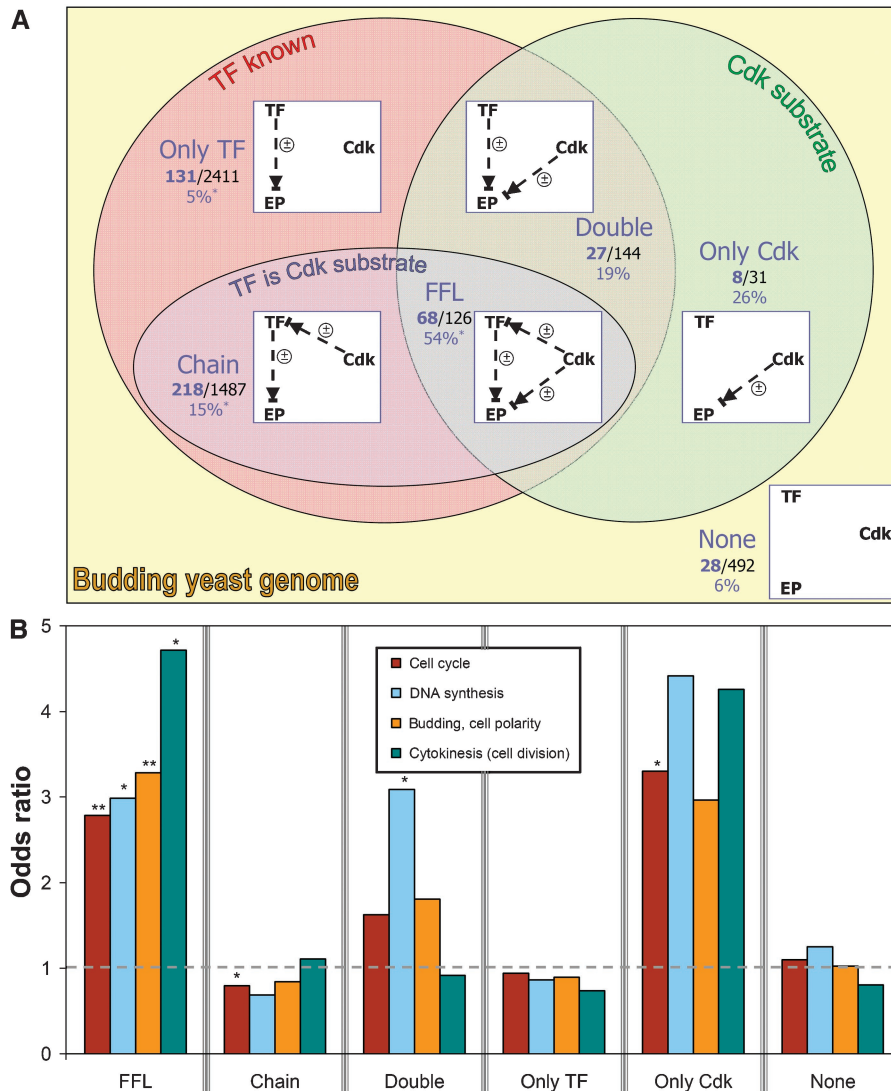
To provide further support for this assertion, we show that cell cycle-related functions are significantly over-represented among the proteins involved in FFLs. We checked the distribution of proteins with cell cycle (and related) MIPS functional category annotations (Ruepp *et al*, 2004; Guldener *et al*, 2005) among the proteins of the different regulatory topologies established on Figure 1A. We found that FFL-regulated proteins are significantly over-represented among

most gene classes with cell cycle functions (Figure 1B; Supplementary Table S3). The converse statement is also true: cell cycle functions are over-represented among the terms associated with FFL-regulated proteins (Supplementary Table S4). Thus, we conclude that FFLs are indeed important transducers of Cdk 'signals' to cell cycle 'responses' (Box 1). The other regulatory topology with high over-representation of cell cycle-related functions is the small group of 'only Cdk'-regulated genes. If our conclusion is correct, then, once the TFs for these genes are discovered, most of these EPs will fall disproportionately into the FFL-regulated group.

If cell cycle EPs are indeed significantly associated with FFL-regulatory topologies, then we must ask what possible function(s) these signal-transduction pathways play in orchestrating progression through the cell cycle. The function of an FFL depends on the signs of the three links of the motif ( $\pm \pm / \pm$ ). The first sign (+ for activation or - for inhibition) indicates the effect of Cdk-mediated phosphorylation on the activity of TF, and the second sign indicates whether the active form of TF upregulates or downregulates gene expression. The product of these two signs indicates the net effect (activation or inhibition) of the 'long arm' of the FFL on EP activity. The third sign indicates whether direct phosphorylation of EP by Cdk activates the protein or inhibits it. The eight possible sign combinations can be divided into two classes (Mangan and Alon, 2003): coherent FFLs, ( $\pm \pm / +$ ) and ( $\mp \pm / -$ ) with the same effective signs on the long and short arms and incoherent FFLs, ( $\pm \pm / -$ ) and ( $\mp \pm / +$ ) with opposite signs. Coherent FFLs have noise-filtering properties (Mangan *et al*, 2003): ( $\pm \pm / +$ ) EPs would be active only when Cdk activity is sustained at a high level (in S + G2 + M phase), and ( $\mp \pm / -$ ) EPs would be active only when Cdk1 activity is absent for a prolonged period of time (in G1 phase). Incoherent FFLs have rich signal response capabilities (Tyson *et al*, 2003; Csikász-Nagy and Soyer, 2008; Kaplan *et al*, 2008). Of particular relevance here, they may respond only to sufficiently strong bursts of a signal: a ( $\mp \pm / +$ ) EP is activated transiently when Cdk activity rises after a prolonged period of low Cdk activity (at the G1/S transition), and a ( $\pm \pm / -$ ) EP is activated transiently when Cdk activity falls after a prolonged period of high Cdk1 activity (at the M/G1 transition). We propose that many of the FFL-regulated proteins identified by our bioinformatics survey of the yeast genome/proteome play exactly these roles in the yeast cell cycle.

To see how FFLs might regulate cell cycle events, we first study their dynamics from a theoretical perspective. We model the eight FFL motifs using ordinary differential equations for phosphorylation reactions and delay differential equations for changes in EP concentrations (Figure 2A; Supplementary Table S5). To implement a single transient activation of EPs per cell cycle, the direct arm of the FFL is expected to have a lower phosphorylation threshold and operate on a faster timescale than the indirect arm. These timescale differences arise naturally in a phosphorylation-transcription FFL: direct phosphorylation of an EP by Cdk happens within seconds, but phosphorylation of its TF has a delayed effect on production of the EP (timescale ~ minutes) (Adelman *et al*, 2002).

Simulation results of the model are shown in Figure 2B. In this figure, we plot (in black) a typical trajectory of



**Figure 1** FFL-regulated proteins are over-represented among both periodically transcribed genes and cell cycle-related genes. **(A)** All verified ORFs of the budding yeast genome were distributed into groups by the topology of their regulation by Cdk (Cdk1) and transcription factors. For each group, we report the number of periodically transcribed/total proteins. For details, see Supplementary Table S1. **(B)** Odds ratios (observed/expected) of finding a gene with a certain type of regulation (as explained in (A)) to be found with an MIPS functional category term given by the colour code in the legend. For detailed statistics, see Supplementary Table S3. On all six panels, a single star denotes those cases where the probability of random appearance (according to a binomial distribution) is less than  $10^{-3}$ , and two stars denotes a probability less than  $10^{-6}$ . The dashed line indicates an expected odds ratio of 1.

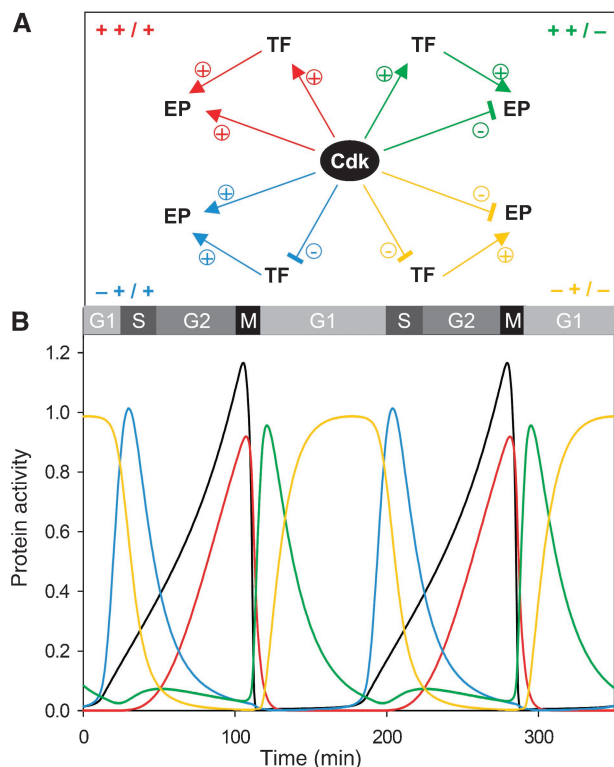
Cdk1–cyclin B during the budding yeast cell cycle. We think of this trajectory as the ‘signal generator’ and the FFLs as ‘signal transducers’ (Box 1). Cdk1–cyclin B activity begins to rise at the G1/S transition, peaks in mitosis and falls rapidly as cells exit mitosis and return to G1 phase. As expected, the coherent FFLs, (– +/–) and (+ +/+), drive sustained EP activity in G1 phase (yellow curve) and in S + G2 + M phase (red curve), respectively. The incoherent FFLs drive bursts of EP activity at the G1/S transition (blue curve: (– +/+) FFL) and at the M/G1 transition (green curve: (+ +/–) FFL). Coherent FFLs ensure the proper temporal appearance of G1-specific and of (S + G2 + M)-specific proteins. Incoherent FFLs convert the periodic rise and fall of Cdk activity into a strict alternation of S-phase entry and M-phase exit, the two transitions that must occur once and only once during each cell cycle to ensure

proper duplication and separation of the cell’s genetic material.

Next, we use diverse evidences to predict, in some cases, the signs of the regulatory effects in our FFL motifs (Supplementary Table S6). From these predictions, we could identify 59 FFLs involving 46 EPs for which the signs of all three links may be proposed (Supplementary Table S7). We found examples of all eight types of FFLs, including some important regulators whose times of appearance in the cell cycle match the predictions of our theory (Figure 2B). In Figure 3, we show examples of an (– +/–) FFL controlling a G1 protein, Sic1 (Knapp *et al*, 1996), an (+ +/+) FFL controlling a mitotic protein, Cdc5 (Zhu *et al*, 2000), an (+ +/–) FFL controlling a cell division protein, Dbf2 (Visintin and Amon, 2001) and an (– +/+) FFL controlling an S-phase initiator, Sld2 (Tanaka

*et al*, 2007a). In the case of Sld2, our database search revealed ‘only Cdk’ regulation (with periodic gene expression). However, Ash1 has been proposed (Teixeira *et al*, 2006) as a potential TF for Sld2. If our theory of signal transduction is correct, then, as Sld2 is an S-phase initiator, the FFL should be (– + / +) and Ash1 is predicted to be an activator of *SLD2* expression. This prediction fits recent experimental results on the role and regulation of Sld2 at S-phase initiation (Tanaka *et al*, 2007b; Zegerman and Diffley, 2007) as well its protein fluctuation profile (not shown) (Masumoto *et al*, 2002).

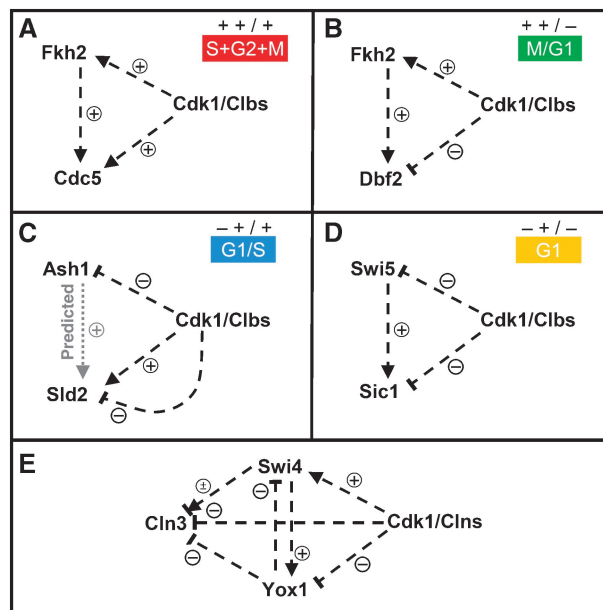
The eight basic FFLs that we have described theoretically are clearly oversimplifications of the signal-transduction schemes operating in real cells. For example, the case of Sld2 (Figure 3C) illustrates that FFLs may be overlapping and even contradictory. Sld2 contains PEST sequences (Supplementary Table S6), which suggests that, after Sld2 is activated by Cdk1 (Zegerman and Diffley, 2007; Tanaka *et al*, 2007b), it is phosphorylated by Cdk1 on a different site that induces its degradation, giving two overlapping, contradictory FFLs. Similar overlapping FFLs might operate for other initiators of DNA replication, such as MCM proteins and Cdc6. (Our methods may be insufficient to identify an early, transient activation of these proteins by Cdk1 before they are degraded.) The case of Cln3 (Figure 3E) suggests that interlocked FFLs may be employed to achieve more complex regulatory effects.



**Figure 2** Four feed-forward loops can regulate the cell cycle. We limit our attention here to the case of upregulation of transcription by TF; for the case of downregulation, see the Supplementary information. (A) Four different types of FFL, for the case where TF upregulates synthesis of EP. Arrows with + or – represent activation or inhibition, respectively. (B) Computer simulations of equations (Supplementary Table S5) describing the interactions diagrammed above. Black line: Cdk activity; coloured lines: EP activities for FFL motifs of same colour in (A). Proposed borders of cell cycle phases are also indicated.

Sic1 (Figure 3D) presents an example where an FFL is composed with a double-negative feedback loop, because Sic1 is a well-known inhibitor of Cdk1-Cln in budding yeast (Schwob *et al*, 1994). The double-negative (=positive) feedback loop functions as a switch, flipping on (Cdk1-Cln activity high) at start and off (Cdk1-Cln activity low) at mitotic exit (Chen *et al*, 2004). By embedding the double-negative feedback loop within a coherent FFL, the switch is made more robust. This feature has been demonstrated recently by removing all Cdk phosphorylation sites from Sic1 (Cross *et al*, 2007), i.e. by removing one leg of the FFL, which made the two transitions less robust. In passing, we note that Sic1 is not an inhibitor of Cdk1-Cln, so the Cln-dependent kinases do indeed control Sic1 by a simple coherent FFL.

Cdc5 (Figure 3A) presents a similar example because of its multiple downstream targets, including proteins such as Cdc25, Wee1 and cyclin B involved in activating Cdk1 at the transition into mitosis (Barr *et al*, 2004). Activation of Cdk1 by Cdc5 turns the coherent FFL into a pair of interlocked positive feedback loops, which may be important in stabilizing M phase. However, it is not clear that this feedback loop is operational in budding yeast, where the functional homologues of Cdc25 and Wee1 do not play such a strong role in mitotic entry.



**Figure 3** Examples of FFLs coupling transcriptional and post-translational controls. Interaction signs ( $\pm$ ) are predicted by the rules presented in Supplementary Table S6. (A) Both the mitotic polo kinase (Cdc5) and its transcriptional activator (Fkh2) are phosphorylated and presumably activated by Cdk1 (bound to B-type cyclins). (B) The mitotic exit initiator Dbf2 shares the same transcription factor (Fkh2) with Cdc5, but Dbf2 appears to be inhibited by Cdk1. Dbf2 has a PEST sequence (Rechsteiner and Rogers, 1996) and its phosphoprotein cannot be detected (Chi *et al*, 2007), suggesting that Cdk1 phosphorylation of Dbf2 induces its degradation. (C) The DNA replication inducer Sld2 is phosphorylated and activated by Cdk1 (Tanaka *et al*, 2007b; Zegerman *et al*, 2007). Although there is no documented TF associated with Sld2, Ash1 has been proposed to regulate *SLD2* expression (Teixeira *et al*, 2006). Our model predicts that Ash1 upregulates production of Sld2. (D) The G1 stabilizer, Sic1, is inhibited by Cdk directly and through its TF, Swi5 (Knapp *et al*, 1996). (E) An example of a complex embedding of FFLs. Further details and other examples in Supplementary Table S7.

We have associated coherent FFLs with EPs that are continually expressed either in G1 phase (when Cdk activity is low) or in S+G2+M phase (when Cdk activity is high). Consulting Figure 1A, we might conclude that ‘only Cdk’ and ‘chain’ topologies can serve these purposes equally well. But theory suggests that coherent FFLs are more robust signal transducers than the single-arm topologies (Mangan and Alon, 2003).

In the case of incoherent FFLs, robustness is not the only advantage: the two regulatory arms are needed to achieve transient activation of the EP. Incoherent FFLs are activated only for a short period of the cell cycle to induce downstream events (DNA replication, budding and cell division) in the correct order. Our analysis revealed that most known FFLs in budding yeast cells are playing roles in these events (Figure 1B) and indeed most examples we predict are incoherent FFLs (Supplementary Table S7). Furthermore, we found examples of DNA replication initiators and cell division inducers that are under direct control of incoherent FFLs (Figure 3B and C).

Altogether, these examples suggest that the eight basic FFLs play important roles in converting periodic Cdk oscillations into a correct temporal sequence of events in the cell cycle, but that these FFLs are often involved in more complex network topologies.

## Conclusion

In all eukaryotic organisms that have been studied in detail, there appear to be two or more Cdk–cyclin pairs that play crucial roles in coordinating cell cycle events. Each one may have its own suite of EPs, probably activated by FFLs. Nonetheless, in fission yeast, a single periodic Cdk–cyclin activity is sufficient to drive all events of the mitotic cell cycle in a viable temporal sequence (Fisher and Nurse, 1996). Our simulation (Figure 2B) shows, in principle, how one Cdk–cyclin pair, utilizing the four basic FFL motifs, can drive G1- and G2-specific proteins and can trigger S-phase entry and M-phase exit in an alternating manner. We imagine that the last common ancestor of present-day eukaryotic cells relied on a single Cdk–cyclin control signal, and that FFLs played a crucial role in converting this single oscillatory signal into coordinated events of a eukaryotic-style cell cycle.

We conclude that the idealized view (Box 1) of FFLs as transducers of periodic Cdk signals provides a reasonable scenario for the evolution of cell cycle controls in early eukaryotes and has merit even now as a ‘first approximation’ of the temporal organization of cell cycle events. In present day organisms, FFLs may be involved in more complex regulatory topologies that exploit and modify their intrinsic dynamical potentials. Nonetheless, incoherent FFLs are still intimately involved in the initiation of DNA synthesis and cell division at the G1/S and M/G1 transitions of budding yeast.

## Materials and methods

### Bioinformatics analysis

Cdk1 substrates were obtained from two large-scale screens (Ubersax *et al*, 2003; Loog and Morgan, 2005). TFs and their targets were downloaded from the YEASTRACT database (Teixeira *et al*, 2006). As many TFs act in complexes, we say that a TF complex is a Cdk1

substrate if at least one of its components is phosphorylated by Cdk1. In total, 600 periodic proteins were identified by de Lichtenberg *et al* (2005). MIPS FunCat annotations of genes were downloaded from the CYGD database (Guldener *et al*, 2005). In the Supplementary information, more details are given on determining the signs of TF–EP connections and of the effect of Cdk1-mediated protein phosphorylations.

### Model construction

We wrote differential equations (Supplementary Table S4) for the rates of change of concentrations of the active forms of TFs and EPs. If Cdk1 directly activates the EP, then we plot the active form of EP only. For cases where Cdk1 inactivates the EP, we assume that phosphorylation induces degradation, thus phosphorylated EP is rapidly degraded, and we plot the total amount of EP as it represents the total active form. Parameters were chosen to get unique EP peaks at different phases of the cell cycle. The Cdk1 time course was generated from a minimal model of the Cdk regulatory system, comparable to (Tyson and Novak, 2001).

### Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website ([www.nature.com/msb](http://www.nature.com/msb)).

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## Conflict of interest

The authors declare that they have no conflict of interest.

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