Phosphorylation network dynamics in the control of cell cycle transitions

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Summary

Fifteen years ago, it was proposed that the cell cycle in fission yeast can be driven by quantitative changes in the activity of a single protein kinase complex comprising a cyclin – namely cyclin B – and cyclin dependent kinase 1 (Cdk1). When its activity is low, Cdk1 triggers the onset of S phase; when its activity level exceeds a specific threshold, it promotes entry into mitosis. This model has redefined our understanding of the essential functional inputs that organize cell cycle progression, and its main principles now appear to be applicable to all eukaryotic cells. But how does a change in the activity of one kinase generate ordered progression through the cell cycle in order to separate DNA replication from mitosis? To answer this question, we must consider the biochemical processes that underlie the phosphorylation of Cdk1 substrates. In this Commentary, we discuss recent findings that have shed light on how the threshold levels of Cdk1 activity that are required for progression through each phase are determined, how an increase in Cdk activity generates directionality in the cell cycle, and why cell cycle transitions are abrupt rather than gradual. These considerations lead to a general quantitative model of cell cycle control, in which opposing kinase and phosphatase activities have an essential role in ensuring dynamic transitions.

Key words: PP2A, Cell cycle, Cyclin-dependent kinase, Phosphatase, Quantitative model

Introduction

Cyclin-dependent protein kinases (Cdks) constitute a family of heterodimeric serine/threonine kinases that require a regulatory cyclin subunit for their activity. Cdks were originally identified as kinases that are essential for triggering mitosis and DNA replication but are now known to also have roles in many other cellular processes. Of the twenty Cdks that have, so far, been identified in humans, Cdk1 is the only one that is essential for the cell cycle in all eukaryotic cells (Malumbres et al., 2009); moreover, it is sufficient to ensure orderly DNA replication and chromosome segregation (Hochegger et al., 2007; Hochegger et al., 2008; Krasinska et al., 2008a; Santamaría et al., 2007).

Regulation of Cdk1 activity involves several separate components that also interact with each other (Fig. 1). Cdk1 is activated by binding one of several cyclins that are sequentially expressed during the cell cycle (Sherr and Roberts, 2004), and directly through Cdk-activating kinase (CAK)-mediated phosphorylation of its conserved kinase-activation loop (Russo et al., 1996). The cyclin subunit also modulates the affinity of the complex towards its substrates (Wohlschlegel et al., 2001). Cyclin degradation, which is initiated by the ubiquitin ligase anaphase promoting complex/cyclosome (APC/C), is crucial for the regulated loss of Cdk1 activity at specific stages during the cell cycle (Murray, 2004). In addition, Cdk1 is inhibited when bound to Cdk inhibitory proteins (CKIs), including Sic1 (in *S. cerevisiae*) and members of the $p21^{Cip1}$ family (in vertebrates). Cdk1 function is also controlled by tyrosine phosphorylation: when phosphorylated on Tyr15 by Wee1 kinase, Cdk1 becomes inactive (Parker et al., 1992); this modification is counteracted by the Cdc25 phosphatases. Of the three Cdc25 isoforms in vertebrates (Cdc25A, Cdc25B and Cdc25C), Cdc25C (hereafter referred to as Cdc25) is abruptly activated at the G2–M transition (Ferguson et al., 2005; Nilsson and Hoffmann, 2000).

In contrast to these well-described mechanisms, the dynamic characteristics of these phosphorylation networks and the way in which the resulting changes in Cdk1 activity control the cell cycle by promoting unidirectional transitions remain poorly understood. In this Commentary, we draw on studies from multiple model systems that are compatible with a quantitative regulation of the cell cycle, and propose a simple general hypothesis that could explain how variations in Cdk1 activity alone can mediate ordered progression through the different phases of the cell cycle. This model does not only place Cdk1 but also those phosphatases that counteract Cdk1 activity at the centre of a network of regulatory enzymes, whose output generates coherent system-wide responses.

Cell cycle control by Cdk1 – the quantitative model

In fission yeast, a single cyclin-B–Cdk1 complex can promote the onset of both S phase and mitosis (Fisher and Nurse, 1996). This observation led to the proposal of the ‘quantitative model’ of cell cycle regulation, whereby different cell cycle events are sequentially triggered by changes in Cdk1 activity (Fisher and Nurse, 1996). Licensing of DNA replication origins in G1 requires Cdk1 activity to be low. This is followed by bulk DNA synthesis in S phase, once Cdk1 activity attains an intermediate level (Diffley, 2004). Finally, once Cdk1 activity exceeds a specific – high – threshold, mitotic onset is triggered (Fig. 2).

More recent studies have lent additional support to this model, whose extreme simplicity still appears valid today. For example, in

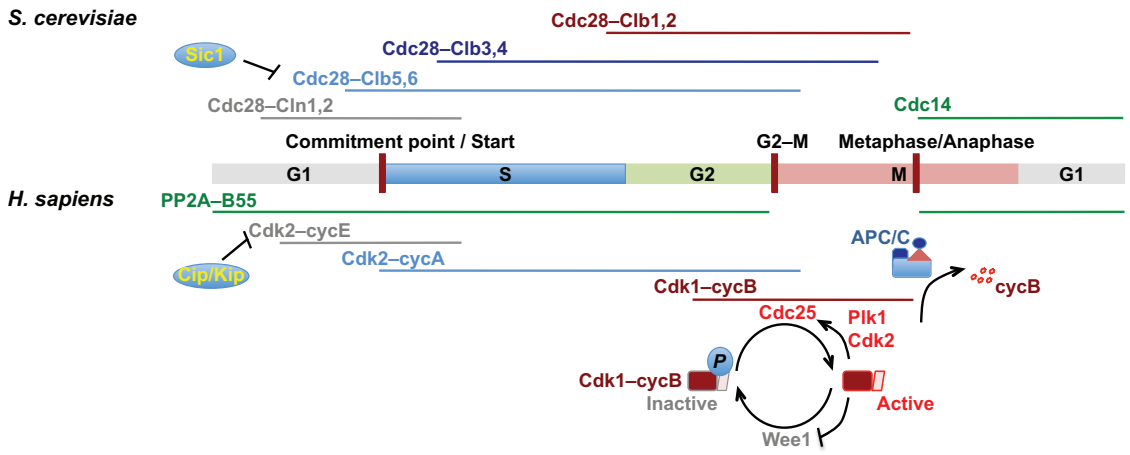


Fig. 1. Cdk and phosphatase regulation during the cell cycle. Comparison of Cdk complexes and phosphatases that control the cell cycle in budding yeast and humans, their respective windows of activity (indicated by straight lines) and other additional regulators. Probable functional homologues are given in the same colour. For more details, please refer to the text.

fission yeast cells that express a constitutively active and non-degradable cyclin-B–Cdk1 fusion protein that has been engineered to be uniquely sensitive to a small-molecule inhibitor, the passage through the entire cell cycle can be artificially governed by changing the concentration of inhibitor so that Cdk1 activity oscillates between low and high thresholds (Coudreuse and Nurse, 2010). In other words, the simple modulation of the activity of a single cyclin-B–Cdk1 combination drives the cell sequentially through S phase and mitosis. Recently, mathematical modelling has demonstrated how this system can recapitulate the wild-type cell cycle (Tyson and Novak, 2011). Moreover, inappropriate alteration of the activity of this complex can even change the order of cell cycle events (Coudreuse and Nurse, 2010). But is the quantitative model universally applicable to all organisms? There are, certainly, similarities between budding and fission yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively). Although three families of cyclins are essential in budding yeast, namely the G1 cyclins Cln1, Cln2 and Cln3, the S-phase cyclins Clb5 and Clb6, and the mitotic cyclins Clb1, Clb2, Clb3 and Clb4, the mitotic Clb2 can – under certain conditions – perform all essential functions of the S-phase and mitotic cyclins (Hu and Aparicio, 2005). It seems, therefore, possible that the correct expression pattern of a single mitotic cyclin might be sufficient to drive budding yeast through the entire cell cycle.

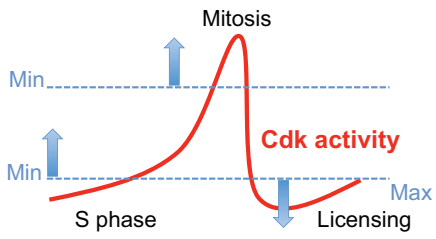


Fig. 2. Cell cycle control by changing Cdk activity – the quantitative model. The vertical axis represents Cdk1 activity. The activity level and history (i.e. ascending or descending activity) determine the cell cycle stage. Thresholds are either minimal (S-phase onset or entry into mitosis do not occur with insufficient kinase activity) or maximal (kinase activity must drop below the threshold to allow replication licensing).

A number of reports have suggested that the quantitative model also underlies cell cycle regulation in metazoans. In mice, for example, Cdk1 is sufficient to control the cell cycle in most cells during early development (Santamaría et al., 2007). Furthermore, both cyclin E genes (*Ccne1* and *Ccne2*) are dispensable for cell cycle progression and are redundant with cyclin A for promoting S phase (Kalaszczynska et al., 2009). In *Xenopus* egg extracts, cyclin A can promote the onset of both S phase and mitosis (Strausfeld et al., 1996), and in human cells, cyclin A can bind to both S-phase and mitotic Cdk substrates (Pagliuca et al., 2011). Strikingly, when cyclin B is relocalised to the nucleus in *Xenopus* egg extracts, it is able to promote DNA replication (Moore et al., 2003), which demonstrates that cyclin B has the intrinsic capacity to drive both main cell cycle transitions. Given these considerations, it is tempting to speculate that manipulation in the timing of protein expression, localisation and levels of either cyclin A or cyclin B might even allow vertebrate cells to operate with a single cyclin-Cdk complex.

However, although these various experimental situations have revealed the principles of cyclin–Cdk redundancy in controlling S phase and mitosis, they obviously do not reflect the physiological situation, in which multiple Cdks and cyclins successively promote these important cell cycle transitions. Furthermore, depletion or knockdown experiments might not lead to the same conclusions as *in situ* experiments in which specific enzymes are inhibited in the presence of all regulators at physiological levels (see Krasinska et al., 2008b for explanation). For example, chemical genetics approaches have shown that – when present – Cdk2 has a rate-limiting role in cell cycle progression in human cells (Merrick et al., 2011) and *Xenopus* egg extracts (Echalier et al., 2012), probably because Cdk2 titrates the nuclear S-phase cyclins A and E. Thus, a single cyclin–Cdk complex might be minimally sufficient, but in a normal *in vivo* situation, when a variety of complexes are present, several of them might become indispensable as a result of the interactions between them.

Moreover, if a single cyclin–Cdk complex suffices – in principle – to promote S phase and mitosis in the correct order, why are multiple complexes involved in all eukaryotes studied to date? One possibility is that this complexity provides robustness by

Box 1. Concepts of cell cycle dynamics

(A) Ultrasensitivity and hysteresis

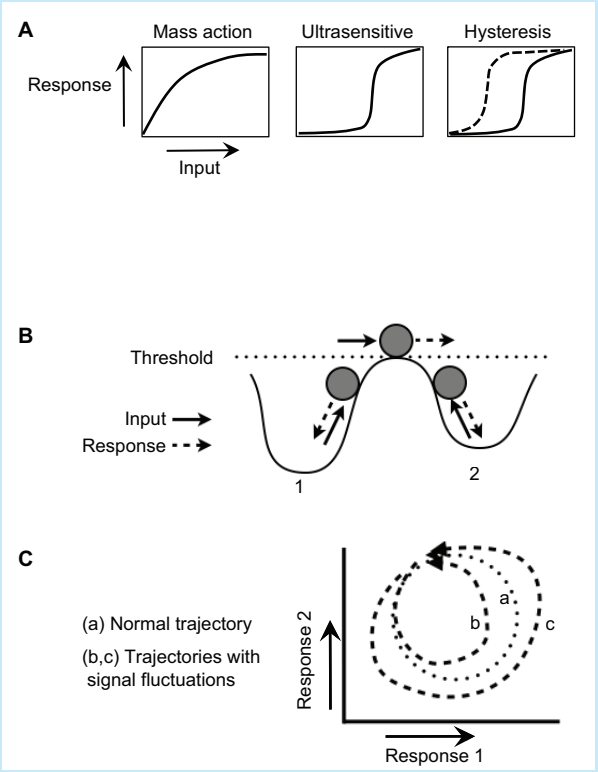
(Left) The curve shows the response of, for example, Michaelis Menten enzyme kinetics, where Input is the substrate concentration and Response the rate of the reaction. (Middle) With cooperativity or other network interactions, the response curve may become ultrasensitive: at low and high inputs it is damped but when a threshold input is reached a switch-like change occurs. (Right) In hysteresis, the response depends on the history of the system. Thus, the forward trajectory (solid line) takes a different path from the reverse trajectory (dotted line). An example is Cdk1 kinase activity (response) to cyclin B concentration (input).

(B) Bistability

Hysteresis can arise from bistability, whereby a system exists only in one of two stable steady states (steady-state 1 and steady-state 2), but can switch between the two. In this analogy, a ball on a hill separating two valleys will always roll back into the valley it came from unless the input provides sufficient momentum to pass the threshold. At the threshold itself, the state is unstable because of noise. An example is Cdk1 activity in the cell cycle.

(C) Limit cycle oscillator

The graph on the right represents all possible states of a system (in a particular dimension) that is cyclic over time, i.e. demonstrates a closed trajectory. The limit cycle (a) is stable because perturbations (b and c), which might be the result of random noise, result in the altered trajectory returning to the limit cycle. This is a property of self-sustaining oscillations, such as that of Cdk1 activity in consecutive cell cycles.



increasing the repertoire of mechanisms that ensure cell cycle order. Indeed, when using the same complex to drive both replication initiation and mitotic onset, perturbations in the control system can more easily lead to genome instability as a result of concurrent DNA replication and mitosis; a situation that can be artificially induced in fission yeast using a single cyclin–Cdk complex (Coudreuse and Nurse, 2010). However, if the system separates the regulation of S phase and mitosis by using distinct complexes with qualitatively different enzymatic activities, then the likelihood of such a deleterious situation arising is reduced.

Finally, a number of studies support the existence of a qualitative level in the cyclin–Cdk system. Although M-phase cyclin–Cdk complexes in budding yeast efficiently phosphorylate most S-phase substrates *in vitro* (Loog and Morgan, 2005), they usually cannot regulate S phase *in vivo* because, unlike Cdk1 in complex with S-phase cyclins, they are efficiently inhibited by Swe1-mediated phosphorylation. By contrast, S-phase cyclin–Cdk complexes only have low activity towards most mitotic substrates in *S. cerevisiae* (Loog and Morgan, 2005). Furthermore, at least *in vitro,* different cyclins also alter the affinity of Cdk for its substrates. For example, although Cdk1, when bound to S-phase cyclins, has a low affinity for mitotic substrates, it retains high affinity for S-phase substrates that contain specific cyclin-binding motifs. Importantly, the analysis of high-affinity binding partners of cyclin A, B and E by time-resolved proteomics in human cells suggests that these principles are also true *in vivo* (Pagliuca et al., 2011). We, therefore, suggest that the co-evolution of cyclins and substrates has superimposed a qualitative dimension to the fundamentally quantitative regulation of the cell cycle by Cdk1 activity, allowing S-phase induction by a kinase complex that cannot trigger mitotic onset.

Biochemical concepts in cell cycle dynamics

Given that oscillations in cyclin-B–Cdk1 activity are sufficient to generate ordered cell cycle progression, two important questions emerge: how are appropriate oscillations in the activity of Cdk1 generated, and how do these, in turn, promote ordered and abrupt cell cycle transitions in a sustained fashion? To understand these issues, one must consider several concepts in regulation dynamics: bistability, hysteresis and ultrasensitivity (see Box 1). Ultrasensitivity means that the response has a sharp input threshold. This generates better dynamic behaviour than Michaelis-Menten kinetics, but is readily reversible, whereas cell cycle transitions are not. Dynamic behaviour can rapidly escape intuitive understanding, and, therefore, mathematical modelling has proven to be essential. Early models on the basis of known interactions of components of the cell cycle network have suggested that mitotic onset is controlled by a bistable switch (Novak and Tyson, 1993). Briefly, bistable systems are characterised by two stable steady states and the absence of an intermediate stable state, and they do not fluctuate in response to small perturbations in input. This robustness relies on hysteresis: once a change in state has been achieved, the initial input is no longer required to maintain the new state, i.e. the response of the system is dependent on its history. Sharp thresholds are also a characteristic of these bistable switches, whereby proportionally small increases in input are buffered until a threshold is reached, whereupon a major change in the output occurs.

Multi-site phosphorylation contributes to ultrasensitivity

As was originally indicated by the autocatalytic amplification of M-phase promoting factor (MPF) in *Xenopus* eggs (Masui and

Markert, 1971) – now often equated to cyclin-B–Cdk1 – the mitotic transition is highly dynamic. This concept has recently been supported by studies investigating Cdk1-dependent phosphorylation in cultured mammalian cells (Gavet and Pines, 2010). Sensitivity is partly the result of positive feedback loops in which Cdk1 stimulates its own activation by promoting dephosphorylation of its conserved inhibitory tyrosine residue, which is targeted by the Wee1 kinase and its counteracting phosphatase Cdc25. It is now known that ultrasensitivity, which contributes to the abrupt changes in Cdk1 activity at the onset of mitosis, is integrated into this system through multi-site phosphorylation of Wee1 and Cdc25 by Cdk1 (Kim and Ferrell, 2007; Trunnell et al., 2011). Indeed, when multi-site phosphorylation events occur by a distributive mechanism, meaning that each phosphorylation requires an independent collision between the kinase and the substrate, the rate of accumulation of those species that are phosphorylated on n residues will vary as a function of the kinase activity to the power of n. If only the highly phosphorylated forms of the substrates have altered functions, low levels of kinase activity are buffered, thereby establishing a threshold of kinase activity.

Interestingly, the same principles appear to govern the G1–S transition in budding yeast through the activation of Clb–Cdk1. In this case, phosphorylation of several of the nine natural Cdk phosphorylation sites on the Cdk1 inhibitor Sic1 allow Sic1 to be recognised and degraded by the ubiquitin ligase Skp, Cullin, F-box containing complex (SCF) (Nash et al., 2001). This could promote ultrasensitivity in the activation of Clb–Cdk1 and threshold-dependent entry into S-phase. However, on the basis of extensive *in vitro* analyses of phosphorylation kinetics, it has recently been proposed that Cdk1-mediated multi-site phosphorylation of Sic1 abides by an ordered processive mechanism, whereby each enzyme–substrate collision leads to phosphorylation of multiple sites. In this model, Cks1, a docking subunit for the cyclin–Cdk1 complex, binds previously phosphorylated, high-affinity Cdk1 sites, thereby allowing Cdk1 to phosphorylate sites of low affinity. This, in turn, leads to recognition of Sic1 by the degradation machinery (Kõivomägi et al., 2011). At first sight, such a mechanism seems to be at odds with the hypothesis that only distributive multi-site phosphorylation of kinase substrates can generate thresholds of kinase activity. Indeed, whereas a distributive process can translate graded inputs into outputs with a sharp threshold (Gunawardena, 2005), a highly processive system cannot do so, because the rate at which the multiple phosphorylated species are generated varies only as the first order of the enzyme concentration. However, the *in vitro* data of Sic1 multi-site phosphorylation suggest that the mechanism is – at least partly – distributive, because substrate competition does occur. Indeed, intermediately phosphorylated species accumulate when the concentration of unphosphorylated substrate is increased (Kõivomägi et al., 2011), which would not occur if the mechanism were strictly processive.

However, multi-site phosphorylation is much more complex than being a simple 'on–off' switch. Recent results from the laboratory of D. R. Kellogg have suggested that, in budding yeast, unphosphorylated Swe1 is inactive, which allows Cdk1 – in complex with mitotic Clbs – to initially escape inhibitory phosphorylation on Tyr19. This complex then binds and phosphorylates Swe1 on a subset of residues, which, in turn, activates Swe1, thereby defining a simple negative feedback loop with regards to Clb–Cdk1 function (Harvey et al., 2011). Only when Swe1 becomes hyperphosphorylated is it inactivated. This generates the well-known double-negative feedback loop that regulates Cdk activity (Fig. 3).

Cdc25, similarly, harbours a plethora of phosphorylation sites, and can also be inhibited and activated by phosphorylation. Phosphorylation of Ser216 promotes the binding of 14-3-3 proteins to Cdc25 and its subsequent sequestration in an inactive state in the cytoplasm. Dephosphorylation of this residue by protein

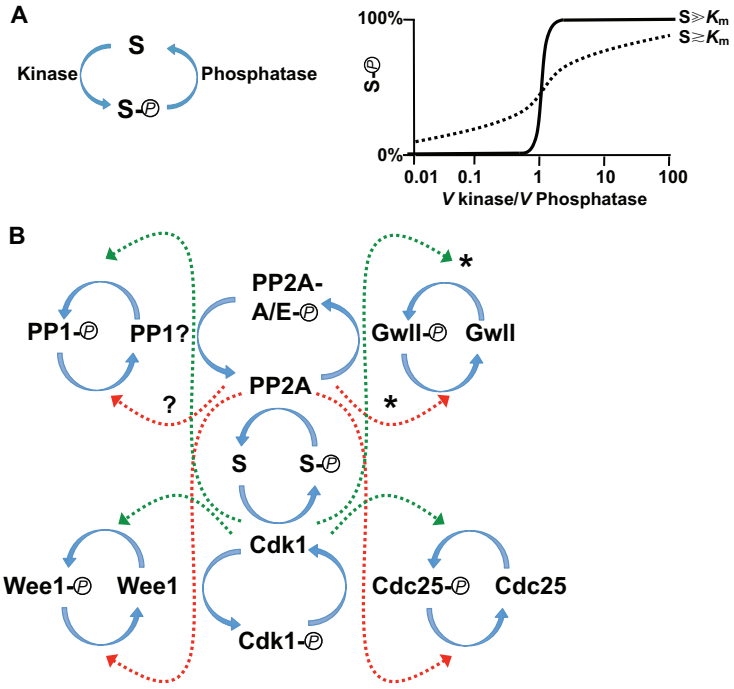


Fig. 3. Kinase-phosphatase cycles can generate sensitivity through multiple mechanisms. (**A**) Left panel: Simultaneous activity of a kinase and a phosphatase constitutes a futile cycle. Right panel: This system can generate sensitivity when the substrate (S) is saturating ($S \gg K_m$). A small increase in the relative kinase:phosphatase activity generates a switch-like change in phosphorylation state; V, velocity (enzyme activity). By contrast, when substrates are limiting ($S \geq K_m$), large changes in this ratio are dampened (dotted line) (**B**) A symmetrical network of kinase–phosphatase cycles in Cdk1 activation and action. Dotted arrows indicate that a substrate of one cycle is an enzyme of another cycle. S, mitotic substrate; A/E, Arpp19 and/or Ensa; PP2A, PP2A-B55; Cdk1, Cdk1–cyclin B; Gwll, Greatwall; ?, hypothesised roles for PP1 in dephosphorylating Arpp19 and/or Ensa, and PP2A in dephosphorylating PP1; *, proposed roles for Cdk1 and PP2A in controlling phosphorylation of Greatwall.

phosphatase 1 (PP1) is promoted by phosphorylation of Thr138 by cyclin-A–Cdk2 and of Thr214 by cyclin-B–Cdk1 (Margolis et al., 2006; Margolis et al., 2003). This prevents the association of 14-3-3 proteins, thereby triggering relocalisation of Cdc25 to the nucleus, where it becomes activated by a multi-site phosphorylation process that is dependent on Cdks and polo-like kinase 1 (PLK1) (Kumagai and Dunphy, 1996; Kumagai and Dunphy, 1999).

Multi-site phosphorylation, thus, appears to be an important and complex feature of Cdk activation, integrating multiple positive and negative regulatory inputs. It might, in fact, govern the functions of Cdk substrates in general, because Cdk phosphorylation sites tend to cluster in disordered regions of proteins but are not precisely conserved throughout evolution (Holt et al., 2009). This is consistent with the idea that individual phosphorylation events might only have minor effects on function, whereas multi-site phosphorylation could generate new protein-binding domains (Holt et al., 2009). Alternatively, as suggested by the analysis of Sic1 multi-site phosphorylation (Kõivomägi et al., 2011), those sites that, in a phosphorylated state, modify protein function (which in the case of Sic1 constitute a phosphodegron motif– a protein domain that, on phosphorylation, serves as a docking site for a specific ubiquitin ligase) might be of low affinity and, thus, inefficiently phosphorylated at low kinase activities. If the mechanism of phosphorylation is – at least partially – distributive, it would create a 'kinase activity buffer' system that could contribute to ultrasensitivity, in which the initial phosphorylation of sites that are efficiently modified would generate high-affinity binding sites for the kinase complex and would, thereby, stimulate the phosphorylation of functional sites.

Kinase–phosphatase cycles and positive feedback loops generate ultrasensitivity

Although multi-site phosphorylation can, in principle, provide a good buffer for low kinase activity, the response that occurs at the kinase activity threshold is not necessarily switch-like (abrupt) but can still be gradual (Gunawardena, 2005). In other words, the system could behave like a rheostat that is set on a minimal threshold. As explained above, one way of making phosphorylation-induced changes more switch-like could be the use of high-affinity sites as 'priming sites' that, when phosphorylated, would increase the affinity of the kinase for lower affinity sites. But, in the same way that friction is required for a car to move, distributive multi-site phosphorylation and positive feedback cannot generate thresholds or switch-like changes in phosphorylation state in the absence of phosphatases that provide resistance to the system.

The simultaneous activity of a kinase and its opposing phosphatase would constitute a futile cycle that buffers changes in enzyme activities and can demonstrate Goldbeter-Koshland kinetics, meaning that the output is sensitive to small variations in the relative activity of the two enzymes at a certain threshold (Goldbeter and Koshland, 1981). This behaviour could allow switch-like changes in the phosphorylation state of their substrates to occur. However, ultrasensitive responses to alterations in the kinase:phosphatase activity ratio only occur when the substrate pool is in excess of both opposing enzymes and when the Michaelis constants (K_m) are low. Because it makes no difference to the dynamics whether the substrate pool is composed of one or many different proteins, and because both S-phase and M-phase Cdk complexes can phosphorylate over a hundred substrates equally well (Loog and Morgan, 2005), such substrate saturation of Cdk1 is likely to be achieved during interphase *in vivo*. Conversely, at the onset of mitosis, the phosphatase – but not Cdk1 – will be saturated with substrates. This system might, therefore, fulfill the requirements for Goldbeter-Koshland kinetics.

The sensitivity in such futile cycles can be improved when kinase and phosphatase are mutually inactivating. This is true for the Wee1–Cdc25 cycle. Wee1 and Cdc25 act upstream of Cdk1 to regulate its activity, and are also downstream effectors of the kinase that are controlled by Cdk1-mediated multi-site phosphorylation: Cdk1 activates Cdc25 (positive feedback) and inhibits Wee1 (double negative feedback). This arrangement, in which feedback loops are combined with an indirect reciprocal inhibition of kinase and phosphatase, is predicted to generate ultrasensitivity in Cdk1 activation and switch-like changes in the phosphorylation state of its downstream substrates (Fig. 3A), which can explain the abruptness of cell cycle transitions. This switch is not triggered below a Cdk1 activity threshold, because multi-site phosphorylation of Wee1 and Cdc25 by Cdk1 is buffered by a Cdk-counteracting phosphatase (Fig. 3B).

Cdk1 is embedded in nested futile cycles, as it is a substrate for one cycle and an enzyme for another (Fig. 3B). In theory, nested futile cycles can considerably amplify the sensitivity of such a system (Goldbeter and Koshland, 1981). However, such cycles alone are not sufficient, and the regulatory interactions between the enzymes involved are important for sensitivity.

Multiple mechanisms promote hysteresis to create directionality

Sensitivity is crucial for generating all-or-nothing transitions, but these new states must also be stable: the cell cycle always proceeds from S phase through G2 and into mitosis. Given that, according to the quantitative model, Cdk1 activity level dictates the cell cycle state, what would happen if fluctuations in activity, for example as a result of noise, occurred around the Cdk threshold for a particular transition? Would the cell keep switching back and forth between, for example, interphase and mitosis? This is indeed technically possible: in HeLa cells expressing a non-degradable cyclin B, mitotic exit can be induced by pharmacological inhibition of Cdk1, but when the inhibitor is removed, cells revert back to the mitotic state (Potapova et al., 2006). Degradation of specific proteins could, in theory, provide a mechanism to ensure irreversibility of such steps. For instance, the degradation of cyclin B would prevent the reversion of G1 cells into mitosis, and the degradation of Sic1 in yeast would prevent reversion of cells from S phase into G1. However, this mechanism is not sufficient. Because degradation mechanisms of cyclin B and Sic1 are triggered by Cdk1-dependent phosphorylation, they can be switched off as the kinase activity disappears, which allows Cdk1 activity to accumulate inside the cell again. Indeed, cyclin destruction is not sufficient for the irreversibility of mitotic exit in yeast. Instead, Sic1 accumulation and activation of APC/C by Cdc20 homologue 1 (Cdh1) build hysteresis into the mitotic exit network (López-Avilés et al., 2009). Hysteresis can, thus, be seen to rely on two mechanisms that have distinct regulation.

The story at mitotic onset is similar. In the original mathematical description based on results obtained from studies using *Xenopus* egg extracts, hysteresis (namely the difference in cyclin B thresholds for activation and inactivation of Cdk1) was

proposed as a mechanism that can generate the unidirectionality of mitotic onset. Hysteresis has since been experimentally shown to occur during the mitotic activation of Cdk1 (Pomerening et al., 2003; Sha et al., 2003). Although hysteresis depends on the feedback loops that involve Cdc25 and Wee1, another system component is also required (Krasinska et al., 2011; Novak and Tyson, 1993).

According to the mathematical model, hysteresis in mitotic onset depends on a phosphatase that counteracts the action of Cdk1 on Wee1 and Cdc25, which has been identified as protein phosphatase 2A (PP2A) (Lee et al., 1991). Indeed, PP2A is capable of reversing the effects of Cdk1-mediated positive feedback through dephosphorylating Cdc25 and Wee1 (Clarke et al., 1993; Mueller et al., 1995). Furthermore, inhibition of PP2A overrides the need for another mitotic kinase, the serine/threonine-protein kinase greatwall (Mastl, herafter referred to as Greatwall), which is normally required for entry into and maintenance of the M-phase state in *Xenopus* (Mochida et al., 2009; Vigneron et al., 2009). PP2A, thus, potentially opposes not only Cdk1 activation but also its activity by dephosphorylating downstream Cdk1 substrates. Conversely, increasing Cdk1 activity leads to a feed-forward loop by indirectly provoking PP2A inhibition through activation of Greatwall and the resulting accumulation of active forms of two highly related PP2A inhibitor proteins, cAMP-regulated phosphoprotein 19 (Arpp19) and alpha-endosulfine (Ensa) (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). Interestingly, Cdk1 can directly phosphorylate Greatwall (Yu et al., 2006) and, thus, Greatwall might also be a substrate for PP2A. This would integrate yet another futile cycle into the network.

The system described above appears to be evolutionarily conserved, because Greatwall is required for mitotic onset in somatic human cells (Burgess et al., 2010), and PP2A-like phosphatases interact with the Cdk1 regulatory network in fission yeast (Kinoshita et al., 1993). Furthermore, genes encoding proteins that are structurally related to Ensa and Greatwall are found in the fission and budding yeast genomes (our unpublished observations). Finally, there is potential for a seventh cycle, because Arpp19 and Ensa are, presumably, also targets for a phosphatase – potentially PP1 as this would make the network symmetrical (Fig. 3B) because Cdk1 can directly inhibit PP1 by phosphorylation (Dohadwala et al., 1994; Yamano et al., 1994). Making the M-phase trigger unidirectional depends on mutual inactivation between Cdk1 and the Cdk-counteracting phosphatase.

In summary, multi-site phosphorylation, positive feedback and feed-forward loops, all of which involve mutually inactivating kinase-phosphatase cycles, are the system components that can explain ultrasensitivity, hysteresis and bistability in mitotic onset, and potentially other cell cycle transitions. The presence of multiple positive feedback loops in Cdk1 activation provides increased robustness with respect to noise, and can give rise to bistable behaviour (Gérard et al., 2012).

Inverse regulation of DNA replication and mitotic onset

The symmetry of the kinase–phosphatase network (Fig. 3B) leads to one interesting prediction: if the cell cycle can be reordered by simply changing Cdk1 activity artificially, then the same should be possible by altering the function of the counteracting phosphatase PP2A. Indeed, mathematical modelling and experiments in *Xenopus* egg extracts have confirmed that Cdk1 and PP2A form a reciprocal network (Krasinska et al., 2011). Imposing a high Cdk1:PP2A activity ratio promotes mitosis even if Cdk1 activity is fixed at a low level by depletion or inhibition. This raises the question of whether DNA replication and mitosis occur simultaneously when the Cdk1:PP2A activity ratio is manipulated at a particular stage of the cell cycle. However, this has been found not to be the case (Krasinska et al., 2011). Multi-site phosphorylation of mitotic regulatory proteins initiates mitosis, and the same mechanism is also employed to inactivate proteins that are involved in DNA replication. Therefore, it seems that, even when bistability is compromised by inhibition of PP2A, the opposing regulation of DNA replication and chromosome segregation by phosphorylation ensures that these two processes do not overlap. Nonetheless, in fission yeast, DNA replication and chromosome segregation can occur simultaneously when Cdk1 activity is artificially increased in cells in G1 phase (Coudreuse and Nurse, 2010). One can speculate that in metazoans, phosphorylation has evolved to achieve a higher level of control over S phase and M phase to ensure a robust separation of these transitions.

Do Cdk–phosphatase networks control all cell cycle transitions?

S-phase onset

Given that a kinase–phosphatase network generates a dynamic transition from interphase to mitosis, one might wonder whether such an organisation underlies other cell cycle transitions. In yeast, it has been proposed that a phosphatase is involved in setting a Cdk threshold for the transition from G1 cyclin–Cdk activity to S-phase cyclin–Cdk activity by opposing the multi-site phosphorylation of Sic1 (Nash et al., 2001). Indeed, the mitotic exit phosphatase Cdc14 can dephosphorylate Sic1 (Visintin et al., 1998) but, at this stage of the cell cycle, Cdc14 is not expected to have an important role, as it is sequestered into the nucleolus, where its inhibitor Net1 resides. Nevertheless, it might be worth investigating whether inactivation of Cdc14 depends on Cln–Cdk1, in which case the network organisation could potentially generate switch-like dynamics in the transition into S phase.

However, so far there is no evidence that a phosphatase opposes Cdk-mediated origin firing. The phosphatase that counteracts phosphorylation of mitotic substrates by Cdk must have a low activity towards S-phase Cdk substrates because they are efficiently phosphorylated, even though overall Cdk activity is low and PP2A is active at the G1–S transition. In the quantitative model, very low Cdk activity might suffice for S phase if there is little or no phosphatase activity against essential S-phase Cdk substrates. Activation of replication itself appears to require phosphorylation of very few Cdk substrates, and the requirement for Cdk activity at this stage can even be overcome in budding yeast when phosphorylation of the two essential S-phase Cdk substrates Sld2 and Sld3 is bypassed (Tanaka et al., 2007; Zegerman and Diffley, 2007). This might also be true in higher eukaryotes because homologues of these proteins exist in vertebrates and are clearly Cdk substrates (Kumagai et al., 2011; Matsuno et al., 2006; Sangrithi et al., 2005; Sansam et al., 2010). It is possible that these few essential S-phase substrates have evolved poor affinity for the mitotic phosphatase that counteracts Cdk activity.

Consistent with this idea, experiments in *Xenopus* egg extracts have not provided any evidence that a Cdk activity threshold is required for DNA replication initiation (Krasinska et al., 2008a).

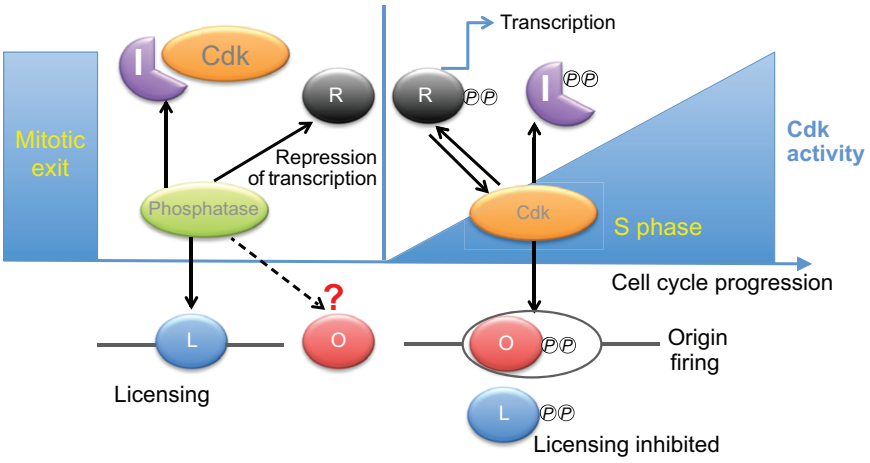


Fig. 4. The Cdk:phosphatase ratio could synchronise the commitment point and onset of S phase. At the point of mitotic exit, Cdk1 activity (blue) drops, and the phosphatase (green) that is a substrate of Cdk1 maintains the activity of transcriptional repressors (R), Cdk inhibitors (I), and replication licensing components (L) by reversing multi-site phosphorylation. Together, these proteins maintain transcriptional repression. Whether or not a phosphatase also acts on origin initiation proteins (O) is currently unknown (?). Passage through the commitment point requires Cdk activation, and results in transcription of cell cycle genes. Cdk activation involves positive feedback as a result of phosphorylation-dependent degradation of inhibitor proteins and cyclin gene transcription. A requirement for multi-site phosphorylation to activate origin initiation proteins might limit their complete activation until cells pass the commitment point.

The apparent Cdk activity threshold at the G1–S transition in yeast and somatic mammalian cells might, however, arise as a result of bistability in G1 transcriptional regulation. In mammalian cells, the commitment (restriction) point is indeed bistable (Yao et al., 2008). Interestingly, the guardians of the restriction point – members of the retinoblastoma (Rb) family – are multiply phosphorylated by G1 Cdks (Cdk in complex with D- and E-type cyclins) and can be dephosphorylated by PP1 (Yan and Mumby, 1999), which is already active at the end of M-phase. Although it is theoretically possible to obtain a bistable switch using a G1 network that does not involve protein phosphatases (Yao et al., 2011), we suggest that the Rb kinase–phosphatase opposition also reinforces bistability at this transition. Once Cdk activity has reached a level that is sufficient to allow cells to pass the commitment point, it would already be high enough to activate replication origins and prevent re-licensing and re-replication. Together, this would result in the synchronisation of passage through the commitment point and replication initiation (Fig. 4). Unlike in yeast and somatic cells, there is no transcription requirement for S phase in *Xenopus* egg extracts and, thus, there may be no need for a Cdk activity threshold to initiate DNA replication.

Mitotic exit

The exit from mitosis also depends on the dynamic control of substrate phosphorylation through competition between activities of phosphatases and kinases. In metazoans, CDK phosphorylation at mitotic exit is probably reversed by PP2A-B55 (Wurzenberger and Gerlich, 2011), which (see above) also prevents Cdk1 activation by controlling Cdc25 and Wee1 and, thereby, creates conditions that are permissive for DNA replication (Krasinska et al., 2011). In budding yeast, Cdc14 can act as a phosphatase for Cdk substrates. There, ordered Cdk-substrate dephosphorylation at the point of mitotic exit depends on the relative catalytic efficiencies of Cdc14 for different substrates, which are partly influenced by substrate affinities (Bouchoux and Uhlmann, 2011).

Conclusions

As discussed above, a mutual inactivation of Cdks and phosphatases that is coupled with multi-site phosphorylation generates ultrasensitivity, creates kinase activity thresholds and hysteresis and, thus, provides the basis for the bistable switch between different cell cycle stages. Considerations of the network dynamics suggest that either modulating Cdk1 activity or PP2A activity is sufficient to order the cell cycle, because the two activities are mutually counteracting, and recent experimental results are consistent with this prediction. To turn a bistable switch into an oscillator requires negative feedback. This is provided by the Cdk1-dependent activation of APC/C, which leads to cyclin B degradation. Combining negative feedback with a bistable switch can create a robust limit-cycle oscillator, which is an oscillator that is self-sustaining because it can return to its normal trajectory following small perturbations in its components (Tsai et al., 2008). Ferrell et al. provide a comprehensive recent review of the network requirements for limit cycle oscillators (Ferrell et al., 2011). Mathematical analysis of the mammalian cell cycle network has shown that sustained oscillations in Cdk1 activity can be obtained with a minimal self-organising system, even in the absence of positive feedback loops between Cdk1 and Wee1 or Cdc25 (Gérard and Goldbeter, 2009; Gérard et al., 2012). Multiple positive feedback loops enhance the robustness and dynamic range of these oscillations (Gérard et al., 2012), even though they are not crucial for the oscillations *per se*. This could explain why they are not essential in fission yeast cell cycles operating on a single Cdk1 complex (Coudreuse and Nurse, 2010).

The quantitative model states that a single cyclin–Cdk complex can generate ordered cell cycle progression with correct timing. To understand how this is achieved, it is essential to consider the kinetic properties of the phosphorylation network, by giving equal consideration to protein phosphatase and kinase activities. Control of the entire cell cycle by a single Cdk complex might be possible because substrates themselves have evolved to have different affinities for mitotic kinases and phosphatases, and the outcome of this competition defines the phosphorylation state of Cdk substrates at a given point in the cell cycle. Nevertheless, current models remain approximate, as they do not take into account the compartmentalisation of enzyme activities within the cell, differences in substrate concentrations *in vivo* and other spatial considerations within living cells. A quantitative description that integrates both spatial and temporal dynamics into a comprehensive model of cell cycle regulation, therefore, remains a major challenge.

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