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4D-networking by mitotic phosphatases

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Faithful progression through mitosis is critically dependent on the timely phosphorylation and dephosphorylation of a host of proteins. The involved protein kinases and phosphatases are embedded in interconnected feedback and feedforward circuits that ensure swift and robust phase transitions. Here we review recent evidence showing that protein phosphatases are modulators of the mitotic entry but also organize the mitotic exit through an orderly dephosphorylation of their substrates. In addition, phosphatases spatiotemporally restrict the phosphorylation of key regulatory proteins and oppose kinases to control highly dynamic mitotic processes, including chromosome congression and checkpoint signaling. In accordance with their important role as nodes in phosphorylation networks, mitotic protein phosphatases are tightly regulated in four dimensions.

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Introduction

All stages of the eukaryotic cell cycle are tightly controlled by protein kinases and phosphatases. However, when it comes to protein phosphatases, most recent progress on their role and regulation is in the context of mitosis, which has prompted us to limit this review to this phase of the cell cycle. Mitosis serves to generate two daughter nuclei with the same sets of chromosomes. The progression through mitosis involves the timely (de)phosphorylation of hundreds of proteins. The major kinases that regulate mitosis are cyclin-dependent kinase 1 (Cdk1), Greatwall (Gwl), Wee1-related kinases, NIMA-related kinases, Polo kinase 1 (Plk1) and Aurora A/B [1]. These and other mitotic kinases are counteracted by members of the structurally unrelated superfamilies of protein tyrosine phosphatases (PTP), haloacid dehalogenases (HAD) and phosphoprotein phosphatases (PPP) [2,3]. The most important mitotic phosphatases are the PTP members Cdc25 and Cdc14, the HAD phosphatase Fcp1, and the PPP members PP1, PP2A and PP6 (Figure 1). Importantly, PPP phosphatases do not exist as free catalytic subunits but each form a diverse set of highly specific holoenzymes through association with one or two noncatalytic subunits that serve as activity regulators, targeting subunits and/or substrate specifiers.

Mitotic protein kinases and phosphatases are implicated in both 'bulk' and 'dynamic' (de)phosphorylation (Figure 1). Bulk phosphorylation is typical for the beginning of mitosis and results in a nearly stoichiometric phosphorylation of proteins on one or multiple sites because the counteracting phosphatases are (largely) inactive [4–6]. In contrast, the mitotic exit is characterized by an ordered bulk dephosphorylation of phosphoproteins, due to drastically increased phosphatase/kinase activity ratios [7••]. Dynamic protein phosphorylation during mitosis is generally associated with low phosphorylation stoichiometries because the involved kinases and phosphatases are simultaneously active, albeit not necessarily at the same location [6].

Here, we review recently acquired insights into the function and regulation of mitotic protein phosphatases. We discuss how mitotic phosphatases are regulated by inhibitors, covalent modifications and subcellular targeting. Some emerging themes are highlighted, including the importance of phosphatase embedment in feedback and feedforward loops, the collaboration between phosphatases and their intrinsic ability to temporally order dephosphorylation events.

Phosphatase regulation by inhibitors

Elegant work in various model systems has revealed that the induction of mitosis depends on the activation of Cdk1 as well as the inhibition of the counteracting PP2A holoenzymes with a B55-type substrate-specifying subunit [8,9,10••,11]. Conversely, the bulk dephosphorylation of Cdk1 substrates at the mitotic exit not only requires the inactivation of Cdk1 but also the reactivation of PP2A-B55 [12]. The phosphatase inhibition at the mitotic entry stems from the Cdk1-mediated activation of Gwl, which phosphorylates the Ensa/Arpp19 proteins to become inhibitors of PP2A-B55 (Figure 2a). The inhibition of PP2A-B55 promotes the mitotic entry in two ways. Firstly, it contributes to the auto-amplification loop of Cdk1 activation by preventing the reversal of Cdk1-mediated phosphorylation of its activity-regulating

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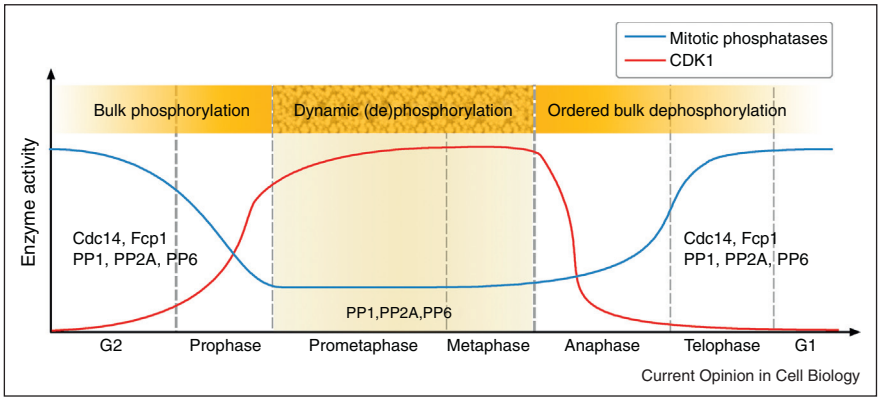
Figure 1



Activity changes of mitotic phosphatases and Cdk1 during mitosis. The mitotic entry is characterized by bulk phosphorylation, which can be explained by the activation of kinases, including Cdk1, and the inactivation of the counteracting phosphatases. During mitosis some proteins are dynamically (de)phosphorylated by opposing kinases and phosphatases. At the mitotic exit key kinases are inactivated and phosphatases are activated, resulting in the ordered dephosphorylation of proteins.

kinases (Wee1/Myt1) and phosphatase (Cdc25). Secondly, the inhibition of PP2A-B55 allows the bulk phosphorylation of Cdk1 substrates at the mitotic entry by blocking their precocious dephosphorylation. The Ensa/Arpp19-mediated inhibition of PP2A-B55 reduces the concentration of Cdk1 needed to enter mitosis by about an order of magnitude [10••]. Surprisingly, the loss of Gwl cannot be completely compensated by increased levels of Cdk1, as this has deleterious effects on spindle assembly. This shows that a tightly regulated balance between Cdk1 and PP2A-B55 is essential for normal progression through mitosis. This balance may imply additional control mechanisms, such as the phosphorylation of the B55 subunit on a Cdk1 consensus site, which hampers PP2A holoenzyme assembly [13]. The combination of these regulatory mechanisms potentially creates a coherent feedforward loop (FFL) (Figure 2a), an established response-delaying module [14,15].

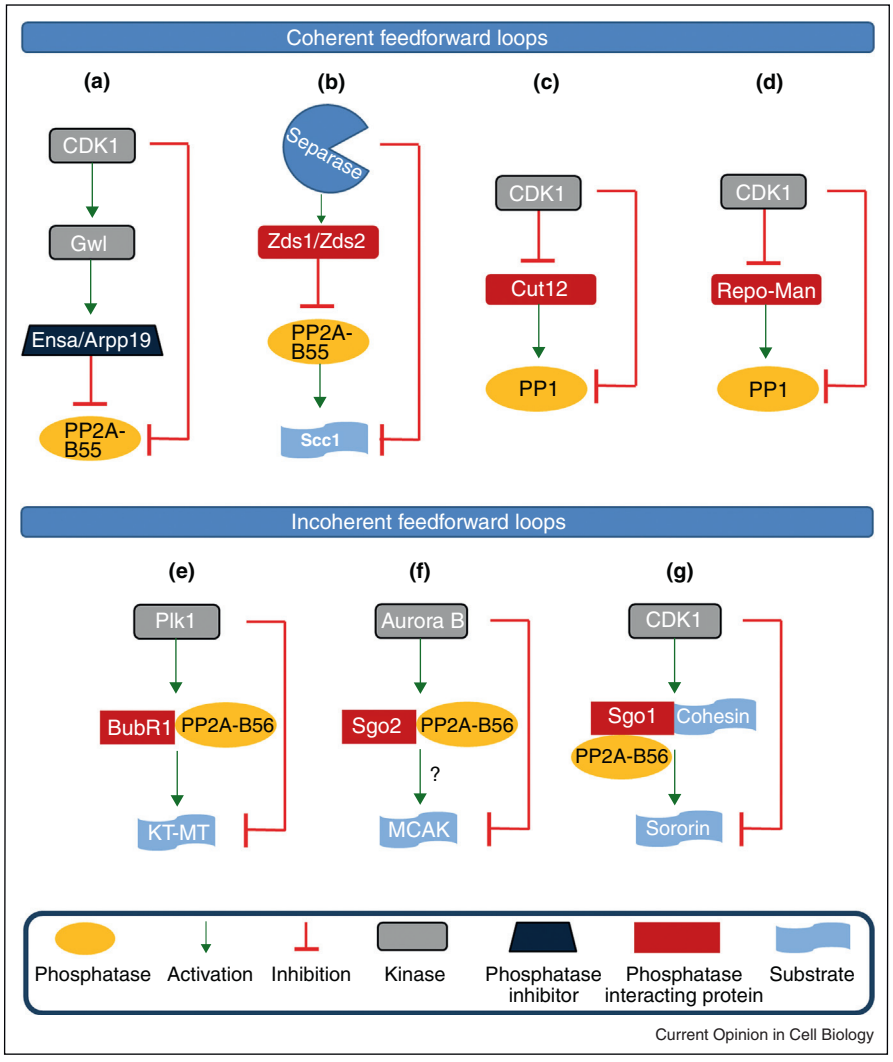
The Gwl-PP2A regulatory unit does not exist in budding yeast, consistent with Cdc14 being the major Cdk1-counteracting phosphatase in this organism [3,16]. Activation of Cdc14 at the mitotic exit depends on the phosphorylation and dissociation of the inhibitor Net1. PP2A-B55 dephosphorylates Net1 and keeps Cdc14 inhibited. Hence, the mitotic function of PP2A-B55 in budding yeast is opposite to that in animals. The mitotic entry function of yeast PP2A-B55 is regulated by the Zds1/2 proteins, which retain a pool of the phosphatase in the cytoplasm [17]. In anaphase the nuclear pool of PP2A-B55 is inhibited by separase in a Zds1/2-dependent manner [18•]. This causes the phosphorylation and separase-catalyzed cleavage of the Scc1 subunit of the cohesin ring that keeps the sister chromatids together at the centromeres (Figure 2b).

The centromeric cohesion of sister chromatids in most animals is maintained by another PP2A holoenzyme (PP2A-B56) that is recruited via Shugoshin (Sgo). During meiosis PP2A-B56 protects centromeric cohesion until (late) metaphase II by keeping the meiosis-specific cohesin subunit Rec8 dephosphorylated. This pool of PP2A-B56 is regulated by Inhibitor 2 of PP2A (I2PP2A), which is also recruited via Sgo [19,20]. I2PP2A co-localizes with Rec8 and PP2A in metaphase II but not before, indicating that the relocalization of I2PP2A is a key determinant of the timely dephosphorylation of Rec8. I2PP2A is also part of a PP2A-B56 network in somatic cells [21], suggesting that it may also regulate centromeric cohesion in mitosis.

Kinase-regulated phosphatase targeting

During the first half of mitosis the assembly of multiple PP1 holoenzymes is opposed by protein kinases. In fission yeast the phosphorylation of Cut12 by Cdk1 and a NIMA-related kinase disrupts its interaction with PP1 and recruits polo kinase, a mediator of the Cdk1-induced auto-amplification loop [22]. Since Cdk1 also directly phosphorylates and inactivates the catalytic subunit of PP1 [2,5], this potentially creates a coherent FFL (Figure 2c). In vertebrates Plk1 indirectly prevents the binding of PP1 to the NIMA-related kinase Nek2A, allowing Nek2A to induce centrosome disjunction by the phosphorylation and displacement of a linker protein [23]. Cdk1 phosphorylates Repo-Man, which reduces the binding of PP1 to this chromosome-targeting subunit [24] (Figure 2d). Finally, the binding of PP1 to the kinetochore proteins KNL1 and CENP-E is prevented until metaphase by phosphorylation of their RVxF-type PP1 docking motif by Aurora kinases [25–28].

Figure 2



Coherent and incoherent feedforward loops. A FFL refers to a network in which a single input modulates a single output via two or more intermediate pathways [15]. In a coherent FFL the intermediate pathways have the same effect, that is, activation or inhibition. In an incoherent FFL the intermediate pathways have an opposite effect, that is, at least one activation and one inhibition. Gwl, Greatwall kinase; KT-MT, kinetochore–microtubule attachment; Sgo1/2, Shugoshin 1/2; MCAK, mitotic centromere-associated kinesin.

In contrast with PP1, the recruitment of PP2A-B56 to mitotic structures is promoted by phosphorylation. During prometaphase PP2A-B56 is dynamically targeted to the kinetochores to counteract Aurora B and stabilize KT–MT attachments [29•]. PP2A-B56 is recruited via a conserved motif of BubR1 and the phosphorylation of this motif by Cdk1 and Plk1 at unattached or tensionless kinetochores, respectively, enhances the B56-BubR1 binding affinity [30••,31,32]. Thus, Plk1 stabilizes KT–MT interactions through recruitment of PP2A-B56. However, Plk1 also recruits destabilizers of KT–MT attachments [33], hinting at the existence of a Plk1-maintained incoherent FFL (Figure 2e) that functions as a pulse-generating module [14]. A B56-interacting 'LSPI'-motif, similar to that of BubR1, has also been identified in Repo-Man, a chromosome-targeting subunit of PP1 [34•]. Repo-Man associated PP2A-B56 dynamically antagonizes Aurora B to promote the histone binding of PP1/Repo-Man during prometaphase.

Vertebrates express two variants of Sgo. The complex of Sgo2 and PP2A-B56 protects centromeric cohesion in meiosis [35]. The assembly of the Sgo2/PP2A-B56 complex depends on the phosphorylation of both Sgo2 and the B56 subunit by Aurora B. However, the phosphorylation of Sgo2 also creates a docking site for the MT depolymerase MCAK, which is itself a substrate for inactivation by Aurora B. Since Sgo2-associated MCAK is a potential substrate for Sgo2-associated PP2A-B56, this may contribute to the establishment of another

incoherent FFL (Figure 2f). Sgo1/PP2A-B56 protects centromeric cohesion in mitosis. Cdk1-phosphorylated Sgo1 binds to cohesin and promotes the dephosphorylation of cohesin-associated sororin by Sgo1/PP2A-B56 [36•]. This maintains centromeric cohesion because sororin competitively prevents the recruitment of the cohesin remover WAPL. Sororin dissociates from cohesin upon phosphorylation by Cdk1. Hence, Cdk1 inhibits cohesion through phosphorylation of sororin on the chromosome arms but maintains centromeric cohesion by potentiating the Sgo1/PP2A-B56-mediated dephosphorylation of sororin (Figure 2g). This incoherent FFL may contribute to the centromeric enrichment of sororin.

Unfertilized eggs of vertebrates are arrested at metaphase of meiosis II by Emi2, an inhibitor of the anaphase promoting complex/cyclosome (APC/C) that targets the Cdk1 co-activator cyclin B for destruction [37]. The stability and inhibitory potency of Emi2 are antagonistically regulated by Cdk1 and PP2A-B56. The phosphorylation of Emi2 by a downstream kinase of the Mos-MAPK pathway results in the recruitment of PP2A-B56, which keeps Emi2 dephosphorylated and the APC/C inhibited. A similar regulation applies to mitosis in early embryos but the recruitment of PP2A-B56 depends here on the phosphorylation of Emi2 by protein kinase A [38].

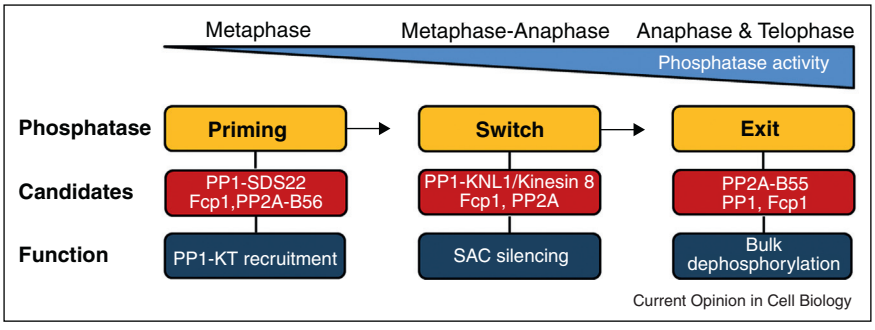
Collaboration between phosphatases

Mitotic phosphatases collaborate by dephosphorylating proteins at distinct levels of a signaling cascade. PP1-Mypt1 protects centromeric cohesion by restraining the activity of Plk1 and Sgo1/PP2A-B56 does so by reversing the Plk1-catalyzed phosphorylation of the cohesin subunit SA2 [39]. PP6 dynamically regulates mitotic spindle formation through dephosphorylation and inactivation of Aurora A [40], whereas PP1 restrains Aurora A by counteracting its activator KIBRA [41]. PP1/Repo-Man antagonizes the chromosomal targeting of Aurora B by removal of its histone H3 docking site and PP2A-B56 contributes to this effect by promoting the histone targeting of Repo-Man [34•].

Phosphatases also work together by sharing substrates in consecutive phases of mitosis. A prime example concerns the MT-KT interface, which is stabilized by BubR1-associated PP2A-B56 in prometaphase and KNL1-associated PP1 in metaphase, and probably involves common substrates [25,29•,30••,42]. The significance of this phosphatase exchange may be that it turns a dynamic, kinase-opposed MT-KT interaction into a more stable interaction that overrules signaling by Cdk1, Aurora B and Plk1.

The arrangement of phosphatases in cascades represents another mechanism of collaboration. One of the key events of metaphase is the dephosphorylation of the PP1-docking motifs of KNL1 (Spc105, Blinkin) and CENP-E by a 'priming' phosphatase, leading to the mass recruitment of PP1 at the kinetochores (Figure 3). Good candidates to dephosphorylate these PP1-docking sites are PP1-Sds22, Fcp1 and PP2A-B56, which are all kinetochore-associated during prometaphase [30••,43,44••,45]. Kinetochore-recruited PP1 functions as a 'switch' phosphatase at the metaphase-anaphase transition in that it triggers the silencing of the spindle assembly checkpoint (SAC) (Figure 3) [42]. The relevant substrates for this APC/C-activation delaying checkpoint are still largely unidentified [46]. In yeast KNL1-associated PP1 dephosphorylates KNL1 itself to disrupt the binding of Bub1/Bub3, two SAC activators [47•]. However, this regulation is not conserved in worms [27] and several additional phosphatase-regulated SAC-silencing mechanisms have been reported. These include the dephosphorylation of the APC/C co-activator Cdc20 by Fcp1 [44••] and PP2A [48], and the dephosphorylation of unidentified substrates by PP1 that is recruited via kinesin-8 [49]. Once the SAC is silenced, the APC/C is activated. This induces anaphase by cleavage of the separase inhibitor securin and inactivates Cdk1 by the degradation of cyclin B. The loss of Cdk1 triggers the

Figure 3



The concept of priming, switch and exit phosphatases. The 'priming' phosphatase(s) dephosphorylate the PP1-binding motif of KNL1 and CENP-E, resulting in the bulk recruitment of PP1 at the kinetochores. 'Switch' phosphatase(s) trigger SAC silencing and 'exit' phosphatases catalyze the ordered bulk dephosphorylation of proteins at the mitotic exit. KT, kinetochore; SAC, spindle assembly checkpoint.

activation of 'exit' phosphatases that catalyze the bulk dephosphorylation of phosphoproteins (Figure 3). For example, PP2A-B55 is reactivated, probably by the dephosphorylation of Ensa/Arpp19 and the B55 subunit, and this catalyzes the bulk dephosphorylation of Cdk1 substrates [13]. Other candidate 'exit' phosphatases are various PP1 holoenzymes (see below) and Fcp1, which is activated by an APC/C dependent mechanism [44••].

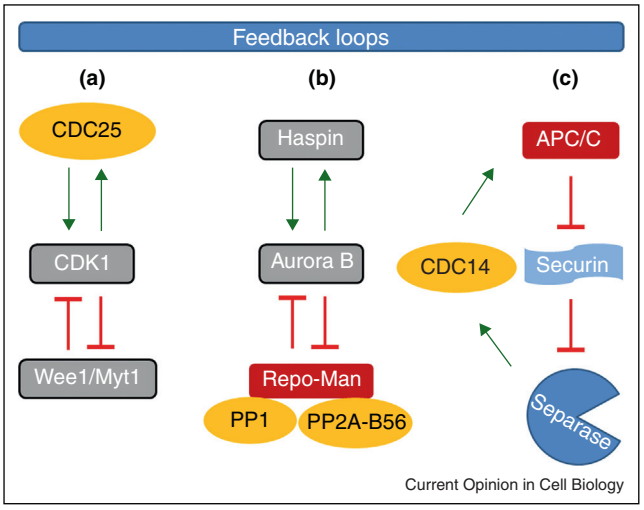
Phosphatase-imposed ordering of dephosphorylation

The ordering of dephosphorylation at the mitotic exit can only partially be accounted for by the sequential activation of phosphatases. In budding yeast Cdc14 dephosphorylates Cdk1 substrates in a defined order, which is explained by a gradually increasing Cdc14/Cdk1 activity ratio and a higher catalytic efficiency of Cdc14 for its early substrates [7••]. Another timing mechanism involves the competition between a mitotic kinase and phosphatase for overlapping substrate-binding sites. Thus, the retinoblastoma protein is only dephosphorylated at the mitotic exit when associated Cdk1 is lost and a docking site for PP1 becomes accessible [50]. The order of dephosphorylation can also be regulated by scaffold proteins that coordinate antagonizing kinases and phosphatases. For example, nuclear-envelope assembly is regulated by Lem-4, which promotes the chromatin binding of BAF through facilitation of PP2A-B55 mediated dephosphorylation and inhibition of the counteracting kinase [51]. There is also emerging evidence for the existence of phosphatase scaffold proteins. Thus, Repo-Man has binding sites for PP2A-B55 (via importin β), PP2A-B56 as well as PP1 [13,24,34•], accounting for the pleiotropic action of Repo-Man in Aurora B targeting, chromosome segregation, chromosome decondensation and nuclear envelope assembly [3,24,52]. The recruitment and/or (in)activation of these phosphatases are likely to be tightly regulated and contribute to the ordered dephosphorylation of their substrates. Another phosphatase that has rather diverse functions during mitosis is PP1-Sds22, which regulates kinetochore-associated Aurora B [43], chromosome segregation [53] and the dephosphorylation of moesin at anaphase to induce polar relaxation [54]. A histone variant in *Tetrahymena i*s fused to a PP1 interacting domain that is similar to Sds22 [55], indicating that PP1-Sds22 may also function in nucleosome organization. The substrate-ordering mechanism for Sds22-PP1 is not known.

Phosphatases in network motifs

Phosphatases are implicated in multiple coherent FFLs that operate during mitotic entry and mitotic exit (Figure 2a–d), when bulk (de)phosphorylation predominates (Figure 1). In contrast, incoherent FFLs function mainly during mid-mitosis (Figure 2e–g), when dynamic (de)phosphorylation is prevalent. Coherent and incoherent FFLs are delay and pulse-generating modules,

Figure 4



Feedback loops involving phosphatases. Cdk1 and Aurora B phosphorylate their regulatory kinases and phosphatases, contributing to their auto-activation and centromeric targeting, respectively. Cdc14 is a component of a positive feedback loop with APC/C and Separase. APC/C, anaphase-promoting complex/cyclosome.

respectively [14]. The significance of coherent FFLs during mitosis may lie in their combination with feedback loops. Thus, the auto-amplification loop for activation of Cdk1 ensures a sharp G2/M-phase transition (Figure 4a) and its combination with a coherent FFL loop between Cdk1 and PP2A-B55 (Figure 2a) serves as a noise filter mechanism to delay the onset of the activation loop until Cdk1 reaches a threshold activity [4]. A similar auto-amplification loop exists for the centromeric targeting of Aurora B, which involves the opposite regulation of a histone H3 kinase and phosphatases (Figure 4b) [34•]. It remains to be seen whether this loop is also connected to a noise-reducing coherent FFL. Finally, Cdc14 is a component of a positive feedback loop with APC/C and Separase that contributes to a swift anaphase transition (Figure 4c). This loop is coupled to a coherent feedforward loop between Separase and Scc1 (Figure 2b) that is predicted to reduce background separase activity [18•]. The importance of incoherent FFLs is that they potentially contribute to 'trial and error' processes during mid-mitosis. For example, Aurora B removes erroneous MT-KT attachments whereas PP2A stabilizes correct attachments. The dynamicity of this system may depend on pulse-generators involving incoherent FFLs with phosphatases (Figure 2e) and/or kinases.

Conclusions and perspectives

It is now well established that mitotic phosphatases are tightly regulated in space and time, and are strongly embedded into interconnected feedback and feedforward loops. However, the regulation of most phosphatases and their interplay with other mitotic kinases and phosphatases

awaits further exploration. It will also be a challenge to map the substrates of specific mitotic phosphatase holoenzymes. Of particular importance will be the identification of the 'priming' and 'switch' phosphatases that induce the mitotic exit. Finally, we suggest that a systems-biological approach can be a powerful tool to generate testable hypotheses on the function and regulation of mitotic phosphatases.

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