Protein phosphatases and the regulation of mitosis

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Summary

Dynamic control of protein phosphorylation is necessary for the regulation of many cellular processes, including mitosis and cytokinesis. Indeed, although the central role of protein kinases is widely appreciated and intensely studied, the importance of protein phosphatases is often overlooked. Recent studies, however, have highlighted the considerable role of protein phosphatases in both the spatial and temporal control of protein kinase activity, and the modulation of substrate phosphorylation. Here, we will focus on recent advances in our understanding of phosphatase structure, and the importance of phosphatase function in the control of mitotic spindle formation, chromosome architecture and cohesion, and cell division.

Key words: Cdk1, Kinase, Mitosis, PP1, PP2A, Phosphatase

Introduction

Post-translational modifications are crucial for the control of the remarkable changes in cellular architecture that are observed as cells enter and exit mitosis. Of central importance among these modifications is protein phosphorylation, which is carried out by a series of conserved serine/threonine protein kinases of the cyclin-dependent kinase (Cdk), Polo, Aurora and Nek families. These protein kinases have well-established roles in phosphorylating key mitotic substrates and have been reviewed extensively elsewhere (Barr et al., 2004; Lindqvist et al., 2009; Nigg, 2001; O’Farrell, 2001; Ruchaud et al., 2007). Protein phosphorylation is typically a short-lived highly dynamic modification and, accordingly, in recent years, equally important roles in mitosis have begun to emerge for specific protein phosphatases (Chen et al., 2007; Gharbi-Ayachi et al., 2010; Kitajima et al., 2006; Mochida et al., 2009; Mochida et al., 2010; Riedel et al., 2006; Zeng et al., 2010). It is this balance of kinase and phosphatase activity that orchestrates the changes seen during cell division. Interference with either type of activity will alter the amount and half-life of substrate phosphorylation (Heinrich et al., 2002), and thus disturb orderly mitotic progression.

This Commentary will focus on a number of recent advances that show the central importance of phosphatase function in the dynamic control of protein phosphorylation during mitosis. The salient structural features of specific phosphatase holoenzymes, which are thought to be important for substrate recognition, will also be described. Particular attention will be paid to recent reports that discuss the regulation of mitotic spindle formation, chromosome architecture and cohesion, and cell division by specific phosphatase holoenzyme complexes.

Introducing the protein phosphatase superfamily

The phosphatases encoded by the human genome can be split into two main groups according to the specific amino acids that they dephosphorylate; these groups can then be further subdivided according to the sequence similarity of their catalytic subunits, sensitivity to inhibitors, and regulatory subunit structure (Lu et al., 2004; Tonks, 2006; Trinkle-Mulcahy and Lamond, 2006). The first major group is the protein tyrosine phosphatase (PTP) family, which includes the dual-specificity tyrosine and serine/threonine phosphatases (DUSPs); these have been reviewed expertly elsewhere (Tonks, 2006). Tyrosine phosphorylation and dephosphorylation have a central role in signal transduction; however, with the notable exception of the phosphorylation and dephosphorylation of tyrosine 15 of Cdk1 by Wee1 and cell-division cycle 25 (Cdc25), respectively, they are of less importance for the regulation of mitosis (Kumagai and Dunphy, 1991; Mueller et al., 1995). Instead, serine/threonine phosphorylation and dephosphorylation are the key regulatory events during cell division. We will therefore focus on this second main group of phosphatases, the serine/threonine-specific phosphatases (PSTPs), and their many roles in mitosis and cytokinesis. PSTPs can be further subdivided into the PPM family of metallo-dependent phosphatases (including PPM1) and the phospho-protein phosphatases (PPP) family, which are both dependent on metal ions for catalysis. PPM family members function in signal transduction and DNA damage pathways (Lu et al., 2004), and it is therefore the PPP family that is most commonly associated with mitotic regulation (Axton et al., 1990; Felix et al., 1990; Picard et al., 1989). Remarkably, brief treatment of cells with the algal toxin okadaic acid, which is specific for PPM enzymes, triggers many of the changes that are associated with mitosis, including chromatin condensation and structural rearrangement of organelles such as the Golgi apparatus (Lucocq et al., 1991; Yamashita et al., 1990). This finding indicates that mitotic entry is normally opposed by PPP enzymes and suggests that inhibition of PPP activity – and not simply protein kinase activation – is required to allow cells to progress into mitosis.

Catalytic mechanisms and inhibitor sensitivity

PSTPs and PTPs use completely different catalytic mechanisms to dephosphorylate their substrates (Barford et al., 1998). Briefly, the PTPs share the conserved active site motif HCX3R, in which the essential catalytic cysteine acts as a nucleophile and forms an intermediate phospho-cysteine during hydrolysis (Barford et al., 1998; Fauman and Saper, 1996). By contrast, the phosphatases of
the PSTP family use two metal ions for catalysis, typically manganese (Mn$^{2+}$) and iron (Fe$^{2+}$), which are coordinated by a set of conserved amino acid residues (Shi, 2009). These bound metal ions coordinate the phosphate group of the substrate and stabilize the negative charge, thus facilitating nucleophilic attack on the phosphorus by a water molecule and hydrolysis of the phosphate ester bond (Goldberg et al., 1995). The catalytic subunits of the PPP family are so similar (Fig. 1A) that all family members are inhibited by the potent algal toxins microcystin-LR and okadaic acid (Fig. 1B), and share three substrate-peptide-binding grooves that lead to the catalytic site (Fig. 1C).

Despite their similarity, the catalytic subunits of PPP family members PP1 and PP2A can be distinguished, to some extent, by their sensitivity to inhibition by okadaic acid. PP2A is inhibited by okadaic acid in the subnanomolar range [half-maximal inhibitory concentration (IC$_{50}$)=0.1 nM], whereas PP1 requires 100-fold higher concentrations of okadaic acid for inhibition (Xing et al., 2006). At a structural level, this can be explained by the absence of specific hydrophobic residues in the catalytic cleft of PP1 that are present in PP2A and are required for tight binding of the hydrophobic end of okadaic acid (Xing et al., 2006). However, although the catalytic subunits of PP1 and PP2A are highly active in isolation, and can be discriminated in terms of toxin sensitivity, they lack appreciable substrate specificity (Agostinis et al., 1992; Imaoka et al., 1983; Mumby et al., 1987). This close similarity and activity of their catalytic subunits are difficult to reconcile with their biological functions and specific substrates observed in cells. It is therefore unsurprising that these catalytic subunits associate with specific regulatory subunits that direct the biological activity of the PPP family phosphatases in living cells. As we will discuss later, this is necessary to explain how PP1 and PP2A regulate unique sets of substrates and cellular events during mitosis.

**Subunit structure of protein phosphatase holoenzymes**

Phosphatases of the PPP family act as multimeric holoenzyme complexes formed from a specific catalytic subunit, which defines the phosphatase (e.g. PP1 or PP2A), and one or more of a number of associated regulatory and scaffolding subunits (Fig. 2A). Phosphatase holoenzyme complexes that contain a PP1 catalytic subunit (C-subunit) associate with a single regulatory or R-subunit, whereas PP2A subfamily enzymes typically possess a scaffolding or A-subunit, in addition to one of the four regulatory B-subunits – B, B’, B” or B‴ (Janssens and Goris, 2001). Other enzymes within the PPP family have a similar subunit composition. PP6 has a similar architecture to PP2A, with an ankyrin repeat domain subunit and a SIT4 phosphatase-associated protein (SAPS) domain scaffolding subunit (Stefansson and Brautigan, 2006; Stefansson et al., 2008), whereas PP4 is thought to possess a single regulatory subunit (Chen et al., 2008). This makes it possible for a relatively small number of catalytic subunits to form a multitude of functionally distinct holoenzymes with unique substrate specificity, regulatory properties and localization within the cell. Binding of these regulatory subunits has the dual role of directing phosphatase activity towards a specific substrate, and also reducing phosphatase activity towards a specific substrate, and also reducing its activity towards other phosphorylated proteins (Agostinis et al., 1992; Imaoka et al., 1983; Mumby et al., 1987). This results in an inhibitory effect towards phosphorylated non-substrate proteins, hence the apparent misnaming of many of these proteins as regulatory inhibitor subunits.

Given the large number of protein kinases and substrate proteins, one would therefore expect equivalent numbers of phosphatase regulatory subunits. Indeed, PP1 is claimed to have in the region of 150–200 ‘validated’ interaction partners (Bollen et al., 2010), which are probably regulatory subunits important for targeting PP1 to diverse subcellular structures and for restricting its specificity (Cohen, 2002). Although many PP1 interaction partners have been described, particularly in the field of mitosis, few have been rigorously validated. It is important to note that PP1 has three potential catalytic subunits, PPP1Cα, β and γ, and that these might form complexes with specific regulatory subunits. The best examples in the context of mitosis are the PP1γ-Repo-man
Protein phosphatases regulating mitosis

Fig. 2. PPP holoenzymes and substrate selectivity. (A) Schematic showing PPP subunit composition. PP1 catalytic subunits (C) associate with a single regulatory subunit (R) drawn from a pool of over 150 potential partners. PP2A has a trimeric structure, with one each of the two possible catalytic and A-subunit variants, and a B, B, B or B subunit. Multiple isoforms of the four B, B, B and B subunits exist. PP4 has a single catalytic and regulatory subunit. PP6 is similar to PP2A, and comprises a single catalytic subunit in conjunction with one each of the ankyrin repeat domain or SAPS domain subunits. For all PPP enzymes, there is a B, B or B, B subunit (R) drawn from a pool of over 150 potential partners. PP2A has a trimeric structure, with one each of the two possible catalytic and A-subunit variants, and a B, B, B or B subunit. Multiple isoforms of the four B, B, B and B subunits exist. PP4 has a single catalytic and regulatory subunit. PP6 is similar to PP2A, and comprises a single catalytic subunit in conjunction with one each of the ankyrin repeat domain or SAPS domain subunits. For all PPP enzymes, there is a

Substrate recognition by protein phosphatase holoenzymes

To date, the crucial question of how substrate selection is achieved has only been addressed by structural studies in a small number of cases (Ragusa et al., 2010; Xu et al., 2008). Two well-studied examples of PP1–regulatory subunit complexes are the PP1–spinophilin complex found in neurons (Ragusa et al., 2010), and the PP1β–MYPT (myosin phosphatase; PPP1R12A) complex that acts as a myosin phosphatase in muscle and is also important in mitosis (Terrak et al., 2004; Yamashiro et al., 2008). Spinophilin, a neuronal PP1α-targeting protein, blocks one of the three potential substrate-binding grooves on the PP1α catalytic subunit (Fig. 2B,C

(CDCA2) holoenzyme and the PP1–PP1R7 (SDS22) holoenzyme (Posch et al., 2010; Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2006).

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and thus allows dephosphorylation of the specific substrate, glutamate-receptor 1 subunit, but not phosphorylase-α, the substrate of a different PP1 holoenzyme (Ragusa et al., 2010). Structural studies have shown two different mechanisms for substrate recognition by PP1, through the binding of regulatory subunits. In the case of spinophilin, the binding site for PP1 is unstructured in solution, but on binding to PP1 it adopts an ordered conformation, which coordinates the catalytic subunit through four distinct regions (Ragusa et al., 2010). Extensive contacts are made between spinophilin and the C-terminal groove of PP1, thus restricting substrate access to the catalytic site, as mentioned above (Fig. 2B). Binding of MYPT again occurs through several extensive sites on PP1β (Terrak et al., 2004), in addition to the RVXF motif of MYPT (Egloff et al., 1997). However, MYPT provides substrate selectivity through the extension of the C-terminal and acidic grooves (Fig. 2B,C).

Similarly, in the case of PP2A, PP2A–Bδ holoenzyme complexes exhibit high phosphatase activity towards the phosphorylated form of the microtubule-stabilising protein tau, whereas PP2A–Bγ holoenzyme complexes have little activity towards the same substrate (Xu et al., 2008). In fact, PP2A–Bγ holoenzymes dephosphorylate tau less well than the PP2A core enzyme devoid of a B-subunit (Xu et al., 2008). In this case, specific substrate recognition is achieved by the interaction of two lysine-rich sequences in the phosphorylated tau substrate protein with an acidic groove in the β-propeller region of the Bδ-subunit (Xu et al., 2008). In PP2A, the position of the substrate-binding B-subunit relative to the catalytic C-subunit is determined by the A-or scaffolding subunit (Fig. 2D). This A-subunit has 15 tandem HEAT (Huntingtin, elongation factor 3, A-subunit of PP2A, TOR1 or scaffolding subunit) 39-amino-acid α-helical repeats that form an L-shaped molecule. The PP2A C-subunits bind to HEAT repeats 11–15, whereas the different B-subunits bind within the first eight HEAT repeats (Fig. 2E). The B'–subunit binds to HEAT repeats 2–8 (Xing et al., 2006; Xu et al., 2006) and also makes contacts with the catalytic subunit (Cho and Xu, 2007), whereas the Bδ-subunit binds to HEAT repeats 1–7 (Xu et al., 2008). This arrangement positions the substrate so that it faces the catalytic cleft of the PP2A holoenzyme. Although no structural data are available for PP6, it is notable that the PP6 scaffolding subunits contain multiple ankyrin repeats (Stefansson et al., 2008). Ankyrin 33-amino-acid α-helical repeats form structures that are comparable to HEAT repeats (Michaela et al., 2002), suggesting that PP6 has a holoenzyme structure that is similar to that of PP2A. These findings provide compelling evidence for the idea that regulatory subunits are the key determinants of phosphatase selectivity, both increasing activity towards specific subunits and reducing activity towards non-substrate phosho-proteins.

Phosphatase holoenzyme regulation

The existence of large holoenzyme complexes raises the question of how they are assembled and regulated. In the case of PP2A, there is evidence that post-translational modifications, including phosphorylation of regulatory subunits and phosphorylation or carboxymethylation of catalytic subunits, might determine holoenzyme composition (Cho and Xu, 2007; Stanевич et al., 2011). The protein kinase Erk prevents assembly of active PP2A–B' holoenzymes by phosphorylating the B' regulatory subunit at serine 337 (Cho and Xu, 2007; Letourneux et al., 2006). As this region of the B'–subunit makes direct contact with the PP2A catalytic subunit, any conformational change is therefore likely to affect this interaction (Fig. 2D). Phosphorylation of PP2A and PP1 catalytic subunits by the tyrosine kinase Src at tyrosine 307 and the mitotic kinase Cdk1 at threonine 320, respectively, inhibits catalytic activity (Chen et al., 1992; Dohadwala et al., 1994; Kwon et al., 1997). In both cases, the phosphorylated residue is close to the C-terminus and phosphorylation in this region is predicted to disrupt the interaction between the catalytic and B-subunits in PP2A (Cho and Xu, 2007).

Other factors implicated in the assembly of functional PP2A holoenzymes are the PP2A phoshatase activator (PTPA) (Fellner et al., 2003) and the ubiquitin-binding c4 protein (Kong et al., 2009; Lenoue-Newton et al., 2011; McConnell et al., 2010; Prickett and Brautigan, 2006). PTPA does not seem to be required for the assembly of holoenzymes, but in its absence PP2A shows reduced activity and stability in cells, suggesting that it has a chaperone-like function and maintains PP2A activity (Fellner et al., 2003). Similar findings have also been reported for c4, although this is not restricted to PP2A, as c4 has been found to interact with the form of PP6 active in mitosis (Prickett and Brautigan, 2006; Zeng et al., 2010). In the case of PP2A, the c4 protein inhibits catalytic subunit ubiquitylation and proteasomal degradation (Lenoue-Newton et al., 2011; McConnell et al., 2010), possibly to ensure that there is a pool of PP2A catalytic subunits available for assembly into functional holoenzymes. Post-translational modifications of both catalytic and regulatory subunits are therefore important considerations, both for the assembly of specific phosphatase holoenzyme complexes and for their subsequent activity during mitosis.

Protein phosphatases and the regulation of mitosis

As outlined above, the requirement for specific protein kinases for the control of entry into and passage through mitosis implies the existence of specific protein phosphatases (Barr et al., 2004; Lindqvist et al., 2009; Nigg, 2001; O’Farrell, 2001; Ruchaud et al., 2007). Here, we will discuss a number of recent examples that highlight the importance of protein phosphatase holoenzyme complexes in controlling both temporal and spatial aspects of protein phosphorylation during mitosis.

Entering mitosis: inhibition of PP2A–Bδ by the Greatwall kinase

Entry into mitosis is driven by the Cdk1 kinase and its associated activator protein cyclin B (Lindqvist et al., 2009; Nigg, 2001; O’Farrell, 2001). Cdk1 is subject to dual regulation. First, the synthesis and stability of cyclin B are controlled such that its levels increase in G2-phase cells, where it can therefore bind to and activate Cdk1. Second, Cdk1 is inhibited by phosphorylation by Wee1 family kinases at threonine 14 and tyrosine 15 (Mueller et al., 1995); these phosphorylations must be removed by the Cdc25 phosphatase for full activity to be achieved (Duphay and Kumagai, 1991; Kumagai and Duphay, 1991). This activation of Cdk1 is generally assumed to be sufficient to drive cells into mitosis, and degradation of cyclin B and Cdk1 inactivation sufficient to allow exit from mitosis (Deibler and Kirschner, 2010; Nigg, 2001; O’Farrell, 2001; Pomerening et al., 2003; Potapova et al., 2006). A number of lines of evidence show, however, that this is only part of the picture, as a specific okadaic-acid-sensitive phosphatase, which dephosphorylates Cdk1 substrates, needs to be inhibited for mitosis to proceed normally (Burgess et al., 2010; Castilho et al., 2009; Vandre and Wills, 1992; Vigneron et al., 2009; Zhao et al., 2011). The protein kinase Erk prevents assembly of active PP2A–Bδ holoenzyme complexes by phosphorylating the B’ regulatory subunit at serine 337 (Cho and Xu, 2007; Letourneux et al., 2006). As this region of the B’–subunit makes direct contact with the PP2A catalytic subunit, any conformational change is therefore likely to affect this interaction (Fig. 2D). Phosphorylation of PP2A and PP1 catalytic subunits by the tyrosine kinase Src at tyrosine 307 and the mitotic kinase Cdk1 at threonine 320, respectively, inhibits catalytic activity (Chen et al., 1992; Dohadwala et al., 1994; Kwon et al., 1997). In both cases, the phosphorylated residue is close to the C-terminus and phosphorylation in this region is predicted to disrupt the interaction between the catalytic and B-subunits in PP2A (Cho and Xu, 2007).

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Protein phosphatases regulating mitosis

Greatwall is a protein kinase that was originally identified in the fruit fly Drosophila melanogaster as a mutant displaying defective chromosome condensation and progression through mitosis (Archambault et al., 2007; Yu et al., 2004); similar findings were also reported for the human homologue microtubule-associated serine/threonine kinase-like enzyme (MASTL) (Burgess et al., 2010; Voets and Wolthuis, 2010). A series of studies has revealed that Greatwall inhibition of PP2A sensitizes cells to Cdk1 activation and can thus promote entry into mitosis (Burgess et al., 2010; Castilho et al., 2009; Lorca et al., 2010; Vigneron et al., 2009; Zhao et al., 2008). Intriguingly, this inhibitory effect is not mediated by direct phosphorylation of PP2A catalytic or regulatory subunits, but, instead, involves two closely related small heat-stable proteins called endosulfine-α (ENSA) and c-AMP-regulated phosphoprotein-19 (ARPP-19) (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). ENSA and ARPP-19 are phosphorylated by Greatwall at serine 67 within a very highly conserved sequence, and can then bind specifically to PP2A holoenzyme complexes that contain Bδ-subunits encoded by the PPP2R2D gene (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). This interaction results in a near-complete inhibition of PP2A–Bδ activity. PP2A–Bδ activity is therefore the inverse of that of Cdk1–cyclin B, and is

Fig. 3. Spatial control of phosphatase function in mitosis. (A) In interphase cells, Cdk1 activity is inhibited, owing to regulatory phosphorylation of threonine 14 and tyrosine 15, and PP2A–B is active. At the G2-M transition, Cdc25 dephosphorylates Cdk1 at these sites, and Cdk1 associates with cyclin B. Cdk1 activates the Greatwall kinase, which phosphorylates the ENSA and ARPP-19 family inhibitors and thereby blocks PP2A–B activity. Cdk1 substrates then accumulate in phosphorylated form. Once the spindle checkpoint is satisfied, the APC/C ubiquitylates cyclin B and it is degraded by the proteasome. ENSA and ARPP-19 inhibition of PP2A–B is relieved and Cdk1 substrates become dephosphorylated. (B) In prophase, Plk1 phosphorylates cohesin complexes and they dissociate from the chromosomes. This is prevented in the centromeric region by a local pool of PP2A–Bδ bound to Sgo1 that maintains the cohesin complexes in a dephosphorylated state and thereby opposes Plk1. (C) Assembly of spindle poles and microtubule organisation requires a set of proteins collectively called spindle assembly factors (SAFs) (Clarke and Zhang, 2008). The activity of these proteins is under the control of a system involving the Aurora A kinase, which is reviewed in detail elsewhere (Barr and Gergely, 2007; Clarke and Zhang, 2008). Aurora A is activated and concentrated at spindle poles by interaction with TPX2, and Aurora A activity is shown by red arrows. This is reversed by the PP6 phosphatase present in the cytoplasm, creating a gradient of Aurora A activity that is high at the spindle and low in the cytoplasm. Aurora A phosphorylation promotes the activity of SAFs involved in pole separation, pole integrity and kinetochore microtubule dynamics. (D) During mitosis, the Golgi apparatus is disassembled through the activity of the Cdk1–cyclin B mitotic kinase, reviewed in detail elsewhere (Lowe and Barr, 2007). In interphase, the Golgi matrix protein GM130 interacts with the p115 vesicle-tethering factor and helps link Golgi membranes and vesicles together. In mitosis, Cdk1–cyclin B phosphorylates GM130 within a basic region that interacts with an acidic patch in p115, thereby disrupting this interaction and linkages between Golgi cisternae. At the onset of anaphase, PP2A–B complexes dephosphorylate GM130, allowing reassembly of linkages between Golgi vesicles and the recreation of ordered stacked Golgi cisternae. The conserved threonine residue in the kinase activation loop is depicted in non-phosphorylated (inactive) and phosphorylated (active) forms by T-OH and T-P, respectively.
high in interphase and low in mitosis (Fig. 3A). Forms of PP2A containing B\(^-\), B\(^+\), and B\(^++\)-subunits are not targets for phosphorylated ENSA and ARPP-19 (Gharbi-Ayachi et al., 2010; Mochida et al., 2010), suggesting that this mode of regulation is highly specific for B-subunits. This is in contrast to inhibitors such as okadaic acid and microcystins, which bind directly to the catalytic subunit of the enzyme and show little specificity for specific PPP family members, as discussed above.

The existing studies differ in their findings on the importance of endogenous ENSA and ARPP-19 in the control of mitotic entry (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). One study reported a role for endogenous ENSA (Mochida et al., 2010), whereas the other shows an exclusive role for ARPP-19 in controlling entry into mitosis (Gharbi-Ayachi et al., 2010). Why two such closely related proteins with similar biochemical function exist is unclear, but it might relate to other regulatory inputs or indicate that redundancy in a crucial cellular pathway controlling mitosis is advantageous. Further studies are therefore needed to clarify the roles of ENSA and ARPP-19. An important consequence of this mechanism is that both the extent and half-life of the pool of Cdk1 phosphorylations normally recognised by PP2A–B\(^\delta\) (McGuinness et al., 2005) is increased (Mochida et al., 2010). This suggests that, during mitosis, these Cdk1 phosphorylation sites are essentially stable, and their rapid or dynamic turnover is only associated with mitotic entry at the G2–M transition and the metaphase–anaphase transition, which follows spindle checkpoint inactivation.

**ENSA and ARPP-19: the first of many mitotic phosphatase inhibitors?**

ENSA and ARPP-19 are not the only phosphorylated phosphatase inhibitory proteins found in cells that might play a role in mitosis. PP1 also has heat-stable inhibitors, including inhibitor-1, a protein of the dopamine- and c-AMP-regulated phosphoprotein family (DARPP-32; PPP1R1A-C) (Hemmings et al., 1984; Wang, X. et al., 2008), nuclear inhibitor of protein phosphatase 1 (NIPP1; PPP1R8), protein kinase C potentiated inhibitor of PP1 (CPI-17; PPP1R14A) and phosphatase holoenzyme inhibitor 1 (PHI-1; PPP1R14B) (Beullens et al., 2000; Elbrecht et al., 1990; Eto, 2009; Huang and Glinsmann, 1975), and inhibitor-2 (PPP1R2), which have high potency when phosphorylated (Li et al., 2007). Like the PP2A inhibitors, there is evidence for holoenzyme specificity and PPP1R14A has selectivity towards PP1–MYPT (PPP1R12A) (Eto et al., 2004), suggesting that it has a function in mitosis. Intriguingly, the PP1 inhibitors also bind to certain protein kinases, and might thereby link kinase activity and phosphatase inhibition. Inhibitor-1 has been shown to regulate Cdk5 activity in neurons (Bibb et al., 1999), whereas inhibitor-2 can directly activate the Aurora A mitotic kinase and is required for normal mitotic progression (Satinover et al., 2004; Wang, W. et al., 2008). Thus, like the Greatwall–ENSA–ARPP-19 pathway, other PP1 inhibitors might function in mitosis to promote a switch-like state change, in which substrate phosphorylation is promoted by simultaneous kinase activation and phosphatase inhibition.

**Cohesion control in mitosis and meiosis: the PP2A–shugoshin pathway**

When cells enter mitosis, DNA condenses to form distinct bodies, the chromosomes. These comprise two sister chromatids that correspond to two discrete copies of the genome. The sister chromatids are linked by proteinaceous multisubunit cohesin complexes that form rings around the two sister chromatids (Nasmyth and Haering, 2009). In mammalian cells, most cohesin is removed from the chromosome arms during prophase by phosphorylation mediated by Polo-like kinase 1 (Plk1) and Aurora B (Hauf et al., 2005). However, cohesin-mediated linkages at the centromeres are protected at this time and thus give mitotic chromosomes their characteristic X-shaped morphology. This arrangement is needed for the attachment of chromosomes to the mitotic spindle, such that one sister chromatid is attached to microtubules that emanate from one pole of the spindle and the paired sister chromatid is linked to the opposite pole. The spindle checkpoint signalling pathway, reviewed extensively elsewhere (Kops, 2008), prevents further progression through mitosis until this geometry is achieved at all chromosomes. How, then, is centromeric cohesion protected? Shugoshin 1 (Sgo1) was originally discovered as a fission yeast factor required to protect the meiotic cohesin protein Rec8 at the centromere from cleavage by separase during anaphase I of meiosis (Kitajima et al., 2004). The fission yeast parologue Sgo2 has a similar role in fission yeast mitosis (Kitajima et al., 2004). Mammals have two shugoshins, Sgo1 and Sgo2, which have discrete functions in mitosis and meiosis, respectively. Sgo2 knock-out mice are viable but infertile, indicating that mammalian Sgo2 is important for meiosis, but not for mitosis (Llano et al., 2008), whereas mammalian Sgo1 is required for mitosis (Salic et al., 2004; Tang et al., 2004). In human cells, Sgo1 localises to centromeres and its depletion results in the premature loss of all sister chromatid cohesion in early mitosis (Kitajima et al., 2005; McGuinness et al., 2005; Salic et al., 2004), which supports the idea that it protects centromeric cohesion during M-phase. The loss of sister chromatid cohesion in human Sgo1-depleted cells can be suppressed by expressing a non-phosphorylatable cohesin SA2 subunit (McGuinness et al., 2005). The explanation for these properties came from the analysis of purified Sgo1 complexes, which were found to contain specific PP2A holoenzymes with B-subunits encoded by the PPP2R5A-E genes (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006). A similar mechanism operates for Sgo2 (Kitajima et al., 2006; Tanno et al., 2010); however, although Sgo1 binds only to assembled PP2A–B\(^-\) holoenzymes, Sgo2 can bind to PP2A catalytic subunits alone, as well as to PP2A holoenzymes (Xu et al., 2009). It is important to note that the interaction of Sgo1 and Sgo2 with PP2A–B\(^-\) complexes does not obviously influence their catalytic activity (Xu et al., 2009), suggesting that they are specific PP2A–B\(^-\) targeting or localisation factors.

Together, these findings suggest that Sgo1 recruits a pool of PP2A–B\(^-\) holoenzymes to the centromeres, where they oppose phosphorylation by Plk1 and Aurora B by dephosphorylating specific substrates (Fig. 3B). This maintains centromeric cohesin in a dephosphorylated state, and thereby prevents the prophase pathway from removing cohesin at centromeres, but not at chromosome arms. A further important point that is highlighted by these findings is the need to simultaneously promote phosphorylation by one pathway, while opposing phosphorylation by another. In mitosis, this problem is solved in the case of the Greatwall–ENSA–ARPP-19 pathway and the shugoshin pathways by their interaction with specific PP2A holoenzymes and either inhibiting them or locally activating them, respectively. As discussed above, it is the specific class of regulatory subunit found in each of these PP2A holoenzymes that imparts these specific regulatory and substrate-binding properties. In this way, phosphorylation of Cdk1 substrates is promoted, whereas, at the same time, phosphorylation of centromeric cohesion complexes is prevented.
Spindle formation: PP1 regulation of Nek2

Formation of a stable bipolar spindle structure is a key step in mitosis and is required for accurate segregation of the chromosomes (Wittmann et al., 2001). The first step in spindle formation, as cells enter mitosis, involves the separation of the duplicated centrosomes to create two independent microtubule-organising centres, which ultimately give rise to the two spindle poles. This centrosome-splitting event is controlled by the Nek2 protein kinase and an opposing okadaic-sensitive phosphatase of the PP1 family (Ghosh et al., 1998; Helps et al., 2000; Meraldi and Nigg, 2001; Mi et al., 2007). PP1γ docks with an RVXF-type motif in Nek2, and has a role in controlling both kinase activity and dephosphorylation of Nek2 substrates, such as the centriolar-linker protein C-Nap1 (Helps et al., 2000). More recent evidence suggests that both PP1α and PP1γ can bind Nek2, but it is PP1α that is more crucial for the regulation of Nek2 activity in vivo (Mi et al., 2007).

Spindle formation: PP6 regulation of Aurora A

Once centrosome splitting has occurred, the centrosomes move apart and their microtubule-organising properties are remodelled to promote bipolar spindle formation. These events are controlled by two main kinases, Aurora A and Plk1, and inhibition or depletion of either kinase leads to monopolar spindles that are unable to mediate chromosome segregation (Girdler et al., 2006; Glover et al., 1995; Lane and Nigg, 1996; Lenart et al., 2007; SUMARA et al., 2004; Sunkel and Glover, 1988). Like many protein kinases, Aurora A and Plk1 are regulated by phosphorylation of the so-called activation or T loop at a conserved threonine residue (Bayliss et al., 2003; Huse and Kuriyan, 2002; Sessa et al., 2005). This phosphorylation event is carried out either in an autocatalytic fashion or by an upstream activating kinase, and results in the correct positioning of key catalytic residues for the phosphotransfer reaction (Huse and Kuriyan, 2002). Targeting and activation of Aurora A and the related kinase Aurora B are accompanied by binding to the activator proteins TPX2 (originally identified as a targeting protein for Xenopus kinesin-like motor protein 2) and inner centromere protein (INCENP), respectively (Bayliss et al., 2003; KUFER et al., 2002; RUCHAUD et al., 2007; Sessa et al., 2005). This interaction protects the phosphorylated T-loop from dephosphorylation and stabilises the active form of the kinase (Bayliss et al., 2003). A prevailing view in the field of mitosis is that mitotic kinases, such as Aurora A, Plk1 and Aurora B, are ‘switched on’ by T-loop phosphorylation upon entry into mitosis and are ‘switched off’, generally by degradation, at the end of mitosis (Barr et al., 2004; Nigg, 2001; Ruchaud et al., 2007). However, such a static model does not take into account that the activity of these mitotic kinases is both spatially and temporally regulated at multiple sites and different times during mitosis, therefore suggesting that there is a highly dynamic equilibrium between kinase T-loop phosphorylation and dephosphorylation.

Surprisingly, until recently, there was little information concerning the identity of the T-loop phosphatases that regulate the major mitotic kinases.

Recent efforts from our own groups have resulted in the identification of the PP2A family phosphatase PP6 as the mitotic T-loop phosphatase for Aurora A–TPX2 complexes in vivo (Zeng et al., 2010). Both PP1 and PP2A catalytic subunits had been previously implicated as regulators of free Aurora A T-loop phosphorylation in vitro (Bayliss et al., 2003; EYERS and MALLER, 2004; Tsai et al., 2003), but our study demonstrates that the PP6 holoenzyme is the major Aurora A–TPX2 T-loop phosphatase in both mitotic extracts and intact mitotic cells (Zeng et al., 2010). Depletion of PP6 catalytic or regulatory subunits, but not PP1 or PP2A, leads to the stabilization of Aurora A T-loop phosphorylation (Zeng et al., 2010). As a consequence, Aurora A is hyperactive, and Aurora-A-regulated spindle assembly factors are misregulated and fail to target to the mitotic spindle (Fig. 3C). This then results in impaired bipolar spindle assembly and defective chromosome segregation (Chen et al., 2007; Zeng et al., 2010). Aurora A overexpression and amplification is considered to be a driving force for tumour formation; therefore, regulation of Aurora A activity by PP6 has relevance beyond the cell biology of mitosis (Bischoff et al., 1998). Depletion of PP6 might have a similar effect to amplification of Aurora A, and, indeed, at least one tumour cell line is known in which both PP6 alleles are mutated (Zeng et al., 2010). Furthermore, loss of PP6 activity, which limits Aurora A kinase activity, might be detrimental when Aurora A is already amplified and might lead to elevated cell death due to mitotic catastrophe.

Spindle formation: PP1 regulation of Aurora B and Plk1

Another specific PPP holoenzyme has been implicated in the regulation of the T-loop of the related kinase Aurora B, which acts in regulating bipolar attachment of chromosomes to the forming mitotic spindle. Similar to Aurora A, Aurora B binds to the activator protein INCENP, which mediates both its activation and targeting to chromosomes (Ruchaud et al., 2007). On the basis of a series of cell biological studies, the PP1–PPP1R7 (Sds22) holoenzyme complex has been suggested to act as the T-loop phosphatase for Aurora B (POSH et al., 2010; SUGIYAMA et al., 2002). Cells depleted of PP1 or PPP1R7 (Sds22) have defects in the regulation of chromosome attachment to the mitotic spindle.

Biochemical analysis showing that PP1–PPP1R7 has T-loop phosphatase activity towards activated Aurora B–INCENP complexes is currently lacking, but, consistent with this idea, cells that are depleted of PPP1R7 have increased Aurora B T-loop phosphorylation (Posch et al., 2010). Similarly, there is evidence that a MYPT-targeted pool of PP1 can influence the activity and level of T-loop phosphorylation of Plk1 (Yamashiro et al., 2008). Plk1 activation and localisation involves docking to phosphorylated binding partners (Barr et al., 2004), and MYPT phosphorylation by Cdk1 in M phase creates such a Plk1-docking site (Yamashiro et al., 2008). It seems counter-intuitive that Plk1 would dock with a protein that would then simply reduce its kinase activity, and it is possible that this interaction might therefore reflect the need to coordinate phosphorylation and dephosphorylation of downstream substrates, rather than of Plk1 itself.

One remaining puzzle relates to how PP6 or PP1–PPP1R7 access the phosphorylated T-loop in activated Aurora A–TPX2 or Aurora B–INCENP complexes. In contrast to most substrates, the T-loop is buried in the kinase active site, rather than being exposed on the surface of the protein. PP6 and PP1–PPP1R7 therefore need to interact with the activated kinase to trigger local unfolding or restructuring of the T-loop peptide, so that it becomes accessible to the phosphatase catalytic site. Once dephosphorylated, the kinase–activator complex would disassemble and dissociate from the phosphatase. Although this is an intriguing speculation, it highlights the need for relevant structures of phosphatase–substrate complexes to be resolved.
**Tension between kinase and phosphatase: Aurora B and PP1**

In addition to direct regulation of Aurora B, PP1 is also involved in the dephosphorylation of Aurora B substrates at kinetochores, thereby stabilizing microtubule attachments at kinetochores under tension (Liu et al., 2010) and helping to promote spindle checkpoint silencing (Meadows et al., 2011; Pinský et al., 2009; Vanoosthuyse and Hardwick, 2009). This is mediated by a pool of PP1γ that directly associates with conserved RVXF and SILK docking motifs in the KNL1 subunit of the microtubule attachment site formed by the KNL1–Mis12–Ndc80 (KMN) network (Liu et al., 2010; Wan et al., 2009; Welburn et al., 2010). In fission yeast, the KNL1 homologue, Spc7, and two additional factors, Klp-5 and Klp-6, which form the kinesin-8 motor complex, bind directly to PP1 and have a similar role (Meadows et al., 2011). In the absence of tension, the centromere-associated pool of Aurora B is in close spatial proximity to the kinetochores and can phosphorylate KNL1 near to the PP1-docking site. This modification prevents docking of PP1 to KNL1 and therefore allows Aurora B substrates to become phosphorylated, which destabilizes microtubule attachments (Liu et al., 2010; Meadows et al., 2011). If microtubule attachment leads to the generation of tension, which stretches the kinetochore away from the centromere, Aurora B can no longer phosphorylate KNL1 (Liu et al., 2010). PP1 can then bind to KNL1 and dephosphorylate Aurora B substrates at kinetochores, thus stabilizing microtubule attachments at kinetochores under tension (Liu et al., 2010).

**Leaving mitosis: PP1 and PP2A clean up after the party**

Once all of the chromosomes have been aligned to form a metaphase plate and the spindle checkpoint is satisfied, the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase modifies its two key substrate proteins – cyclin B and the separase inhibitor securin. Cdk1 inactivation by cyclin B degradation allows exit from mitosis and entry into anaphase, whereas separase cleaves cohesin linkages at centromeres and allows sister chromatid separation in anaphase (Musacchio and Salmon, 2007). This is insufficient to allow exit from mitosis, as the mitotic phosphorylation carried out by Cdk1 and other kinases still needs to be reversed. In budding yeast, this is carried out by the Cdc14 phosphatase, whose activity is under the control of a complex signalling pathway, the mitotic exit network (Stegmeier and Amon, 2004). Although there is some evidence that Cdc14 has a role in anaphase regulation and cytokinesis in multicellular eukaryotes, it is unlikely to be the major phosphatase controlling Cdk1 substrate dephosphorylation. Recent evidence indicates that vertebrate Cdc14 has a more important function in DNA repair than in mitotic dephosphorylation (Mishima et al., 2004; Neef et al., 2009). The role of phosphorylation in the assembly and disassembly of the Golgi apparatus is well understood, and a number of key substrates for mitotic kinases and phosphatases have been identified, as reviewed elsewhere (Lowe and Barr, 2007). In the case of one of these substrates, the Golgi matrix protein and vesicle-tethering factor GM130, dephosphorylation during anaphase at a conserved Cdk1 phosphorylation site has been shown to involve the PP2Aγ (PPP2R2A) holoenzymes (Fig. 3D) (Lowe et al., 2000; Lowe et al., 1998). Intriguingly, this is the same PP2A holoenzyme class than sister chromatid separation and for local control of phosphatase activity. The PP1γ–Repo-man (CDCA2) pathway, which regulates chromatin architecture in anaphase, provides an example of the need for local control (Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2006). Cdk1–cyclin B negatively regulates Repo-man by direct phosphorylation until the onset of anaphase, when Repo-man becomes dephosphorylated and then targets PP1γ to chromatin (Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2006). In chicken B-cell lines that lack condensin complexes, chromosomes lose their structure during anaphase, which results in abnormal mitosis (Vagnarelli et al., 2006). This defect can be suppressed by depletion of Repo-man or overexpression of a B-type cyclin (Vagnarelli et al., 2006). The authors of this paper suggest that an as yet unidentified activity, which they term regulator of chromatin architecture (RCA), is inactivated, and presumably dephosphorylated, by PP1γ–Repo-man. More recent evidence indicates that one substrate of the Repo-man pathway is the form of histone H3 that is phosphorylated by the protein kinase haspin at threonine 3 (Qian et al., 2011). Threonine-3-phosphorylated histone H3 is found predominantly at centromeres, where it forms the chromosome binding site for the survivin subunit of the Aurora B chromosomal passenger complex (Dai et al., 2006; Kelly et al., 2010; Wang et al., 2010). A Repo-man-bound pool of PP1γ actively dephosphorylates histone H3 at threonine 3 during metaphase and as cells exit mitosis, and prevents the spread of Aurora B away from the centromeres and onto the chromosome arms (Qian et al., 2011).

Although globally increased phosphatase activity is important for the removal of the phosphorylations carried out by Cdk1–cyclin kinase, if anaphase is to occur, other protein kinases must remain active and have essential functions in the later events of cell division. Both Plk1 and Aurora B have to phosphorylate substrates that are required for cell contractility and the proper timing of cytokinesis (Bastos and Barr, 2010; Douglas et al., 2010; Neef et al., 2007; Neef et al., 2006; Petronczki et al., 2007; Wolfe et al., 2009). The activity of a number of these substrates is inhibited by Cdk1 and activated by Aurora B and Plk1 phosphorylation, respectively (Mishima et al., 2004; Neef et al., 2007; Toure et al., 2008). It is therefore essential that the phosphatase holoenzymes that remove the phosphorylation carried out by Cdk1, Plk1 and Aurora B during mitosis do not act on Plk1 or Aurora B phosphorylation sites that are required for anaphase and cytokinesis. Although a clearer picture has begun to emerge of the importance of phosphatase holoenzyme complexes that act on different classes of phosphorylation site for the proper regulation of mitosis and cytokinesis, many details remain to be resolved.

**Membrane organelles: rebuilt behind the Greatwall**

Cells exiting mitosis also need to reasssemble organelles of the secretory and endocytic pathways, as well as segregate the chromosomes and rebuild an interphase nucleus (Lowe and Barr, 2007). The role of phosphorylation in the assembly and disassembly of the Golgi apparatus is well understood, and a number of key substrates for mitotic kinases and phosphatases have been identified, as reviewed elsewhere (Lowe and Barr, 2007). In the case of one of these substrates, the Golgi matrix protein and vesicle-tethering factor GM130, dephosphorylation during anaphase at a conserved Cdk1 phosphorylation site has been shown to involve the PP2A–βγ (PPP2R2A) holoenzymes (Fig. 3D) (Lowe et al., 2000; Lowe et al., 1998). Intriguingly, this is the same PP2A holoenzyme class
that is inhibited by Greatwall (Gharbi-Ayachi et al., 2010; Mochida et al., 2010; Schmitz et al., 2010), suggesting that this pathway also contributes to Golgi disassembly and reassembly in mitosis. Importantly, neither PP2A–Bα (PPP2R5A) holoenzymes nor PP1 complexes can dephosphorylate GM130 (Lowe et al., 2000). Once GM130 is dephosphorylated, Golgi membrane vesicles become tethered together and self-organise to form a stacked structure that is characteristic of interphase cells (Lowe et al., 2000; Lowe et al., 1998; Shorter and Warren, 1999).

**A dynamic future for protein phosphatases in mitosis**

Emerging evidence has started to provide a clearer picture of how substrate phosphorylation is controlled during mitosis. Multiple inhibitory mechanisms exist to reduce the activity of some PP1 and PP2A holoenzymes towards their substrates. Importantly, this inhibition is coordinated with the activation of mitotic kinases such as Cdk1–cyclin B. As a consequence, phosphorylation of these substrates will increase during prophase and metaphase. Furthermore, these phosphorylations will be essentially stable during this time (long half-life), owing to the absence of specific phosphatase activity (summarised in Fig. 4A). Dynamic turnover of these sites will occur only during the mitotic entry and exit transitions. Simultaneously, pools of other phosphatase holoenzymes remain active during mitosis and might even be concentrated at specific cellular structures. A pool of PP2A–B’ is concentrated at centromeres, where it reverses Plk1 phosphorylation of cohesin complexes and thereby prevents their dissociation from chromatin. Cohesin at chromatin arms will thus not be protected and is subsequently phosphorylated and displaced (Fig. 4B).

Although there has been a large amount of progress in our understanding of phospho-regulation and the role of specific protein phosphatases in mitosis, many questions remain. Phosphatase regulators have been identified for a number of crucial pathways in mitosis and, because of the known importance of dynamic phosphorylation in other pathways, many more must remain to be identified. Examples include spindle checkpoint control, centrosome and microtubule function, cell shape and adhesion, and central spindle control during cytokinesis. In this Commentary, we have stressed the importance of the identification and verification of phosphatase holoenzyme complexes that are linked to discrete biological functions. Phosphatase catalytic subunits do not act alone, and it is unlikely that useful information can be obtained using them in isolation in vivo or in vitro. As discussed

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**Fig. 4. Temporal control of phosphorylation site dynamics by coordinated kinase and phosphatase activity.** (A) The levels and activity of key kinase and phosphatase regulators of mitosis. Cdk1, cyclin B, PP1, PP2A–Bδ and the Greatwall pathway are depicted, together with the extent and half-life of protein phosphorylation. (B) PP2A–B’ remains active during mitosis and is concentrated at centromeres, where it protects cohesins from Plk1 activity.
here, there are also spatial and temporal considerations – simply put, is the phosphatase active at the right time and in the right place to dephosphorylate the substrate in question (Figs 3 and 4)? Caution is especially necessary when interpreting in vitro data, because, although a particular enzyme might dephosphorylate a given substrate, it might not actually be active at the appropriate time and place within cells.

The discovery of the agal toxins okadaic acid and microcystin created much excitement (Bialojan and Takai, 1988; Cohen et al., 1990; MacKintosh et al., 1990), but, as discussed in this Commentary, this was tempered by the realisation that they show little specificity for individual PPP family members. For this and other reasons, PPP enzymes have not been viewed as promising targets for drugs or small inhibitory molecules. This view has started to change, owing to a number of discoveries. First, there is the identification of highly specific cellular inhibitors of PP2A holoenzymes, which have added to the known group of specific PP1 inhibitors. Second, a small compound called guanabenz can be used to disrupt specific PP1–PPP1R15A holoenzyme complexes (Tsay et al., 2011). Structures of PP2A holoenzymes bound to ENSA and ARPP-19 inhibitors, or PPP1R15A bound to guanabenz, will therefore provide valuable insight into how holoenzyme-specific inhibition can be achieved. These principles should prove useful to aid the development of specific phosphatase inhibitor compounds and drugs with a multitude of potential uses, both in the laboratory and in the clinic.

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35
Protein phosphatases regulating mitosis

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