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REVIEW

Protein phosphatases in the regulation of mitosis

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The accurate segregation of genetic material to daughter cells during mitosis depends on the precise coordination and regulation of hundreds of proteins by dynamic phosphorylation. Mitotic kinases are major regulators of protein function, but equally important are protein phosphatases that balance their actions, their coordinated activity being essential for accurate chromosome segregation. Phosphoprotein phosphatases (PPPs) that dephosphorylate phosphoserine and phosphothreonine residues are increasingly understood as essential regulators of mitosis. In contrast to kinases, the lack of a pronounced peptide-binding cleft on the catalytic subunit of PPPs suggests that these enzymes are unlikely to be specific. However, recent exciting insights into how mitotic PPPs recognize specific substrates have revealed that they are as specific as kinases. Furthermore, the activities of PPPs are tightly controlled at many levels to ensure that they are active only at the proper time and place. Here, I will discuss substrate selection and regulation of mitotic PPPs focusing mainly on animal cells and explore how these actions control mitosis, as well as important unanswered questions.

Dynamic phosphorylations control cell division

Mitosis is characterized by an ordered series of events in which first the nuclear envelope breaks down, chromosomes compact, and the mitotic spindle starts to assemble. Once the kinetochores on sister chromatids are attached to the mitotic spindle and properly bioriented, anaphase is initiated, and the sister chromatids separate and move to opposite poles of the dividing cell. This is followed by the reassembly of the nuclear envelope, decompaction of chromatin, cytokinesis, and finally, abscission that separates the two new daughter cells (Fig. 1 A). Because translation and transcription are suppressed during mitosis, the post-translational modification of proteins plays a prominent role in the orchestration of mitosis (Taylor, 1960; Prescott and Bender, 1962). Cdk1 in complex with cyclin B1 is the major mitotic kinase phosphorylating thousands of Ser-Pro (SP) and Thr-Pro (TP) sites to initiate and regulate mitosis (Olsen et al., 2010; Petrone et al., 2016). Cdk1 activity is controlled by the regulation of cyclin B1 stability, with cyclin B1 being degraded at metaphase by the anaphase-promoting complex/cyclosome (APC/C) in complex with Cdc20 (Pines, 2011). APC/C-Cdc20 activity is inhibited by the spindle assembly checkpoint (SAC) such that APC/C-Cdc20 becomes active only once all microtubules have properly attached to the kinetochores (Lara-Gonzalez et al., 2012). In addition to Cdk1-cyclin B1, many other mitotic kinases, including Plk1, Mps1, Bub1, Haspin, and the Aurora kinases, regulate cell division (Kettenbach et al., 2011; Santamaria et al., 2011). These kinases have unique localization patterns and phosphorylate distinct, specific sites on target proteins. However, kinases alone are insufficient to control dynamic processes such as mitosis because the phosphorylation of serine and threonine residues is extremely stable, with the half-life likely being longer than the lifetime of our planet (Lad et al., 2003). Therefore, protein phosphatases ensure that phosphorylations are dynamic and responsive. This is illustrated by the fact that cells are unable to exit mitosis when Cdk1 is inhibited if protein phosphatase activity is blocked (Skoufias et al., 2007). Because there are roughly 10 times more serine/threonine kinases encoded in the genome compared with serine/threonine phosphatases (Manning et al., 2002; Moorhead et al., 2007; Chen et al., 2017), this raises the question of how this limited number of phosphatases can balance the activities of all the kinases. As will be discussed, the solution to this problem is the dynamic assembly of phosphatase catalytic subunits into multiple different holoenzymes that target distinct substrates.

Phosphoprotein phosphatases (PPPs) regulating mitosis

Genetic screens, as well as cell-based and biochemical assays, have revealed that members of the PPPs namely PP1, PP2A, and PP6 holoenzymes, are important and essential regulators of mitosis in many model organisms (Ohkura et al., 1988; Booher and Beach, 1989; Doonan and Morris, 1989; Kinoshita et al., 1990; Mayer-Jaekel et al., 1993; Goshima et al., 2003; Chen et al., 2007; Afshar et al., 2010; Manchado et al., 2010; Schmitz et al., 2010; Zeng et al., 2010; Wurzenberger et al., 2012). In addition, Cdc25 phosphatases control mitotic entry, and Cdc14 is the major mitotic exit phosphatase in budding yeast (Stegmeier and Amon,

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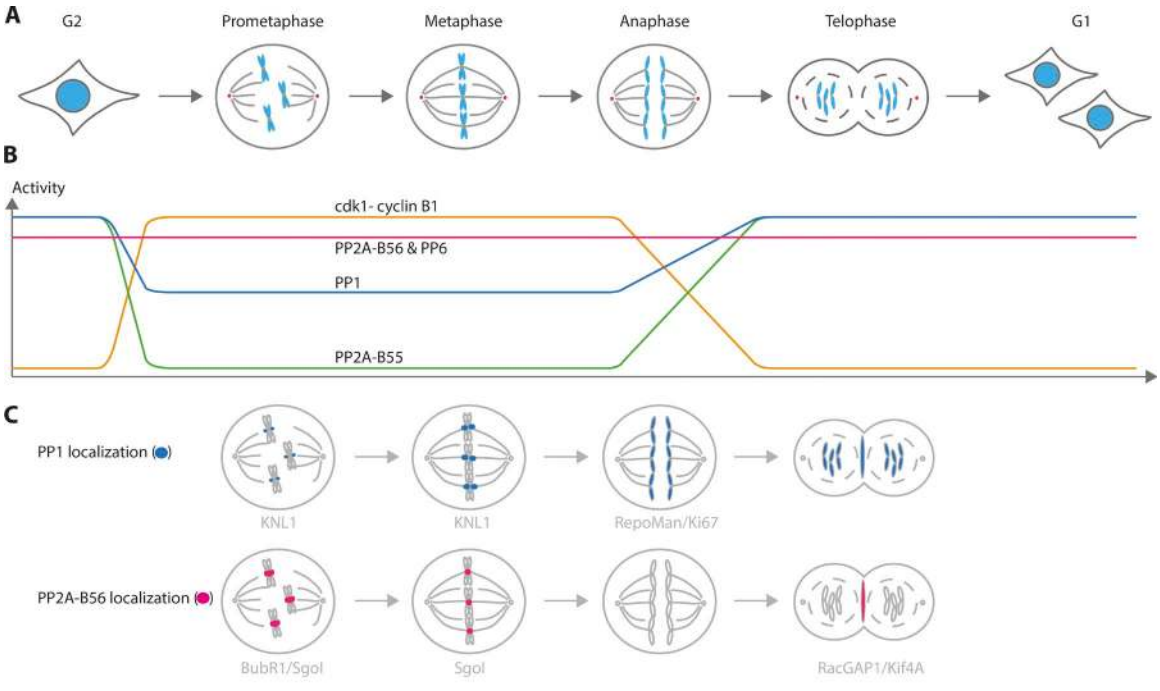

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Copy number estimate of mitotic phosphatase components (Log10)

PP1CA	5,7	PPP2CA	6,1	PP6C	5,8
PP1CB	6,2	PPP2CB	4,7	PP6R3	5,2
PP1CC	5,1	PPP2R1A	6,3	ANR28	5,1
		PPP2R1B	4,8		
		B55α	5,6		
		B56δ	5,6		

Figure 1. **Cell division and the activity and localization of mitotic phosphatases. (A)** An overview of the different stages of mitosis and the movement of chromosomes. **(B)** Activity profile of mitotic phosphatases and Cdk1 in relation to mitotic progression. To a large extent, these activity profiles are hypothetical and will depend on substrate and localization. **(C)** Localization patterns of PP1 (blue) and PP2A-B56 complexes (red) during cell division in human cells. **(D)** Copy number estimates of mitotic phosphatase components based on proteomic data from HeLa cells (Bekker-Jensen et al., 2017). For simplicity, only the isoform with the highest expression level is shown for B55, B56, PPP6R, and ANR subunits.

2004; Boutros et al., 2006; Clifford et al., 2008; Lindqvist et al., 2009). This function of Cdc14 is not conserved, and instead, PPP members are important regulators of mitotic exit in many other organisms. The focus of this review will be on PP1, PP2A, and PP6 because they are well-established regulators of mitosis, but it should be pointed out that Calcineurin (PP2B) is an important regulator of meiosis (Mochida and Hunt, 2007).

PP1 isoforms regulating mitosis

At first glance, PP1 appears to be the simplest mitotic phosphatase in that it consists of only a catalytic subunit (Fig. 2 A). However, PP1 likely never exists in an unbound form but assembles into hundreds of different holoenzyme complexes that each have distinct substrate-binding domains and localization patterns (Moorhead et al., 2008; Hendrickx et al., 2009; Heroes et al., 2013; Choy et al., 2014). The three human isoforms of PP1 (PPP1CA-C, PP1α-γ; there are two splice variants of PP1γ: PP1γ1 and PP1γ2, with PP1γ1 often referred to as PP1γ) differ mainly in the amino acid sequence of their C-terminal extension (Peti et al., 2013). PP1α and PP1γ display the highest sequence similarity and exhibit a distinct localization pattern during mitosis compared with PP1β (Fig. 1 C; Andreassen et al., 1998; Trinkle-Mulcahy et al., 2003). Biochemical and genetic data have shown that PP1 counteracts the activity of Cdk1, Aurora B, and Mps1 and regulates Plk1 activity (Francisco et al., 1994; Wang et al., 2008; Yamashiro et al., 2008; Wu et al., 2009; London et al., 2012; Nijenhuis et al., 2014).

PP2A in complex with specific B subunits control different aspects of mitosis

The PP2A active holoenzyme is a trimeric complex composed of a catalytic subunit (PP2ACα-β and PPP2CA-B), scaffolding A subunits (Aα-β, PR65A-B, and PPP2R1A-B), and one of four regulatory subunits: B (B55, PR55, and PPP2R2A-D), B′ (B56, PR61, and PPP2R5A-E), B″ (PR48/PR70/PR130 and PPP2R3A-C), and B‴ (Striatins or PR93/PR110) (Janssens and Goris, 2001; Shi, 2009). The 65-kD scaffolding A subunit is horseshoe shaped, and through its N-terminus, it interacts with the regulatory subunits while its C-terminus binds PP2AC (Fig. 2, B and C). The PP2AC-A complex is abundant in the cell while the regulatory subunits are rate-limiting for the formation of holoenzymes (Fig. 1 D; Bekker-Jensen et al., 2017). It is the PP2A-B55 and PP2A-B56 complexes that appear to be the major PP2A complexes regulating mitosis; however, they perform very distinct functions. PP2A-B55

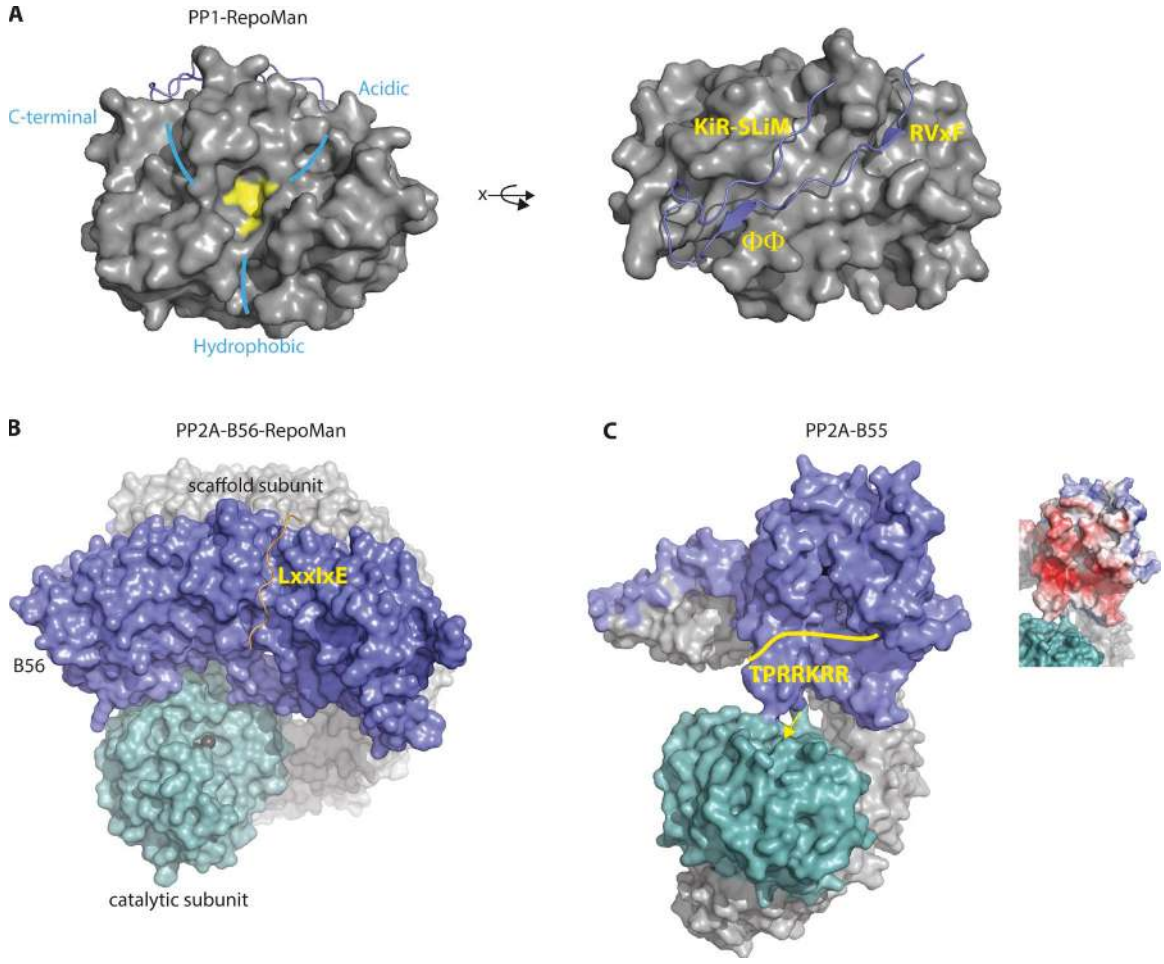

Figure 2. **Structural aspects of mitotic phosphatases and binding to SLiMs. (A)** Structure of PP1 in complex with RepoMan. The active site (yellow), the three possible substrate-binding grooves around the active site (light blue), and the binding of RepoMan motifs to different pockets on PP1 are indicated. **(B)** Model of PP2A-B56 bound to the LxxIxE motif of RepoMan with catalytic subunit (turquoise), scaffold (gray), and B56 (blue). **(C)** Structure of PP2A-B55 with the hypothetical binding of a basic region to the acidic region on the B55 subunit. The electrostatic potential of the B55 surface is shown on the right with basic residues in red. The yellow arrow indicates the active site.

counteracts Cdk1 activity to induce mitotic exit, whereas its activity is suppressed during the earlier stages of mitosis (Fig. 1 B; Castilho et al., 2009; Mochida et al., 2009; Schmitz et al., 2010; Cundell et al., 2016). PP2A-B56 associates with different mitotic structures and counteracts several mitotic kinases, such as Aurora B and Plk1 (Fig. 1 C; Foley et al., 2011; Suijkerbuijk et al., 2012; Hertz et al., 2016). B55 subunits are largely composed of a WD40 domain with an acidic surface facing toward the catalytic subunit. B56 subunits are composed of 15 tetratricopeptide repeats forming a horseshoe-shaped structure (Fig. 2, B and C; Xu et al., 2006, 2008; Cho and Xu, 2007). The four isoforms of B55 (B55α–δ) and five isoforms of B56 (B56α–ε) appear to be redundant, although expression levels of the different isoforms vary in different cell types and the B56 isoforms display distinct localization patterns (Foley et al., 2011; Bastos et al., 2014).

PP6, the least understood mitotic phosphatase

The PP6 holoenzyme is a trimeric complex composed of PP6C bound to one of three Sit4-associated proteins (SAPS) domain–containing subunits (PPP6R1-3 and SAPS 1–3) and one of three ankyrin repeat domain subunits (ANR28, ANR44, and ANR52; Luke et al., 1996; Stefansson et al., 2008; Guergnon et al., 2009; Zeng et al., 2010). The PPP6R subunits act as platforms for assembling the trimeric holoenzyme, and in yeast, the active complex is likely to be a dimer of PP6C and PPP6R because ANR subunits are not present in yeast (Guergnon et al., 2009). The N-terminal SAPS domain binds PP6C while a possibly unstructured C-terminal region binds an ANR subunit. The ANR subunits are predicted to be largely α helical in nature, similar to other ankyrin repeat proteins (Mosavi et al., 2004). PP6 controls Aurora A activity by dephosphorylating the T-loop during mitosis and counteracts casein kinase 2 (CK2; Zeng et al., 2010; Rusin et al., 2017).

While the mitotic phosphatases have very different compositions, they share a very similar catalytic subunit, the properties of which are discussed below.

PPP active site specificity or lack thereof

The structure of the PP1 catalytic subunit reveals that the catalytic domain of the PPP family is a compact, extremely conserved ∼35-kD structure with little variation in the residues in and

surrounding the active site (Fig. 2 A; Egloff et al., 1995; Goldberg et al., 1995). The catalytic domain is a metalloenzyme with two metal ions bound in the active site that coordinate the phosphate group of the substrate and activate a water molecule for an in-line attack on the phosphate (Egloff et al., 1995; Goldberg et al., 1995; Zhang et al., 1996; Zhang and Lee, 1997; Swingle et al., 2004). PPPs are extremely efficient in catalysis; estimated to increase the rate of hydrolysis by a factor of 10^{21}, they are one of the most efficient enzymes known (Swingle et al., 2004). The structures of PPP catalytic subunits reveal the absence of a clear peptide-binding cleft in the active site. There is, instead, an open surface with three putative spacious substrate-binding clefts that radiate from the catalytic center in a Y-shape: the C-terminal, hydrophobic, and acidic substrate-binding grooves (Fig. 2 A; Egloff et al., 1995; Goldberg et al., 1995). The structure of a mitotic PPP catalytic domain in complex with a substrate is not currently known. However, the structure of the catalytic domain of PP5, which is very similar to PP1 in structure, bound to a Cdc37 phosphomimetic peptide has been solved, as well as structures of PP2B and PP1 bound to phosphate (Griffith et al., 1995; Choy et al., 2014; Oberoi et al., 2016). The PP5-Cdc37 structure reveals that the substrate conformation is largely dictated by interactions between PP5 and the peptide backbone and that side chains of the substrate engage in water-mediated interactions with PP5, allowing the accommodation of a large array of side chains. In agreement with the PP5-Cdc37 structure, the sequence alignment of the known substrates of different PPPs reveals little sequence specificity beyond the actual phosphorylated residue (Li et al., 2013). This explains why PPPs can counteract multiple kinases but does not rule out that some sequence preference exists. As an example, in vitro assays with model peptides have shown that proline residues located C-terminally to the phosphorylation site is not a favorable circumstance for rapid dephosphorylation (Agostinis et al., 1987, 1990, 1992).

The use of isolated PPP enzymes in in vitro assays might poorly represent specificity because their association with specific binding partners controls specificity in vivo. An illustration of this is the structure of PP1 in complex with Mypt1 or Spinophilin (Terrak et al., 2004; Ragusa et al., 2010). In both instances, the PP1 active site does not undergo conformational changes; rather, Mypt1 reshapes the region around the active site by modulating its electrostatic properties while Spinophilin occupies the C-terminal cleft, thereby preventing the binding of substrates that rely on this groove. This suggests that many of the PP1 holoenzymes have unique substrate preferences despite the same active site. Whether this principle applies to other mitotic phosphatases is unknown and will require further structural and functional characterization.

Another feature of the PPP active site is a distinct preference for phosphothreonine over phosphoserine, an effect most clearly observed with PP2A but likely applicable to all PPP family members (Pinna et al., 1976; Agostinis et al., 1987; Deana and Pinna, 1988; Donella-Deana et al., 1990, 1994). The molecular basis for this phosphothreonine preference is not known, but a combined effect of a lower Km and higher Kcat on phosphothreonine substrates is recognized (Agostinis et al., 1987; Hein et al., 2017). It is possible that features of the active site contact the additional methyl group on threonine. Additionally, in vitro data also suggest that the nature of the metal ions in the active site influences this preference, but whether this is relevant in vivo is unclear (Agostinis et al., 1987). High-resolution structures of PPP-substrate complexes are needed to address this. As discussed later, this difference in the kinetics of phosphoserine and phosphothreonine is important for the orchestration of temporal events during mitosis and during the cell cycle in budding yeast (McCloy et al., 2015; Cundell et al., 2016; Godfrey et al., 2017; Hein et al., 2017). Many kinases also display preferences for phosphorylating either serine or threonine, and this will further influence the dynamics of a phosphorylation site (Chen et al., 2014).

It is evident from the structural and functional analysis of PPPs that the active site contributes a minimum of substrate specificity. So how is specificity achieved? As indicated, the substrate specificity of PPPs is achieved through the formation of a large number of holoenzymes the assembly of which I will discuss next.

Short linear-interaction motifs (SLiMs) control PP1 holoenzyme formation

How do PPPs assemble into multiple holoenzymes? An emerging theme is that distinct binding grooves on the catalytic subunit or binding pockets on the B regulatory subunits of PP2A recognize SLiMs in the unstructured regions of binding partners. These binding partners can be direct substrates, localize PPPs to specific mitotic structures for local dephosphorylation, or recruit specific substrates to the PPP. SLiMs are typically 4–10 amino acids long with two or three residues acting as core binding determinants and mediate low micromolar affinity interactions with globular domains (Tompa et al., 2014; Davey et al., 2015). A hallmark of SLiMs is that they are degenerate, thereby allowing a spectrum of affinities that can fine-tune signaling pathways; this is also the case for SLiMs binding to phosphatases. SLiMs control PPP specificity at multiple levels, for instance, by recruiting the phosphatase directly to a substrate, localizing it to a specific cellular compartment, or mediating the binding of an inhibitor or regulator to it.

One of the first SLiMs reported to bind a PPP family member was the RVxF motif that binds to a hydrophobic binding pocket on PP1 at a site distinct from the active site (Fig. 2 A; Egloff et al., 1997; Terrak et al., 2004). The RVxF motif is present in the vast majority of PP1-interacting proteins, and the motif is best described as (K/R)-(K/R)-(V/I)-(FIMYDP)-(F/W) (Wakula et al., 2003; Meiselbach et al., 2006; Moorhead et al., 2008; Hendrickx et al., 2009). These PP1-binding motifs are used to target PP1 to multiple proteins during mitosis, for example, the kinetochore protein Knl1 to regulate chromosome segregation and SAC silencing (Liu et al., 2010; Meadows et al., 2011; Nijenhuis et al., 2014), inhibitor 1 and 2 to regulate PP1 activity (Hurley et al., 2007; Marsh et al., 2010), Mypt1 to regulate Plk1 activity (Yamashiro et al., 2008; Matsumura et al., 2011; Dumitru et al., 2017), Kif18A to regulate chromosome oscillations (Häfner et al., 2014), and RepoMan and Ki67 to control chromosome decompaction and dephosphorylation of chromatin-associated factors (Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2011; Booth et al., 2014). A conserved interactor of PP1 is Sds22, which might

act as a chaperone for PP1 holoenzymes because it binds to a distinct surface of PP1 without interfering with RVxF interactions (Ceulemans et al., 2002). However, the exact function of Sds22 and its effect on PP1 holoenzyme activity remain unclear, making it difficult to interpret the reported mitotic phenotypes of Sds22 removal (Ohkura and Yanagida, 1991; Peggie et al., 2002; Posch et al., 2010; Wurzenberger et al., 2012; Eiteneuer et al., 2014; Rodrigues et al., 2015).

How is the dynamic distribution of PP1 among all these binding partners controlled if the vast majority engages the RVxF-binding pocket on PP1? In several RVxF motifs, the x position is a phosphorylation site for Aurora kinases, which can consequently prevent the association of PP1 with the motif and thereby regulate PP1 holoenzyme formation (Nasa et al., 2018). For instance, the RVSF motif in the kinetochore protein Knl1 is phosphorylated by Aurora B, thereby dampening PP1 binding to kinetochores until microtubule attachment (Liu et al., 2010; Bajaj et al., 2018). Another distinct mechanism of phosphoregulation is the PP1 extended binding region of RepoMan (Fig. 2 A) that contains multiple Cdk1 phosphorylation sites, which prevent PP1 binding until anaphase (Qian et al., 2015). In addition to the RVxF motif, further motifs (e.g., SILK, ΦΦ, KiR-SLiM) have been described that bind to distinct grooves on PP1 (Hendrickx et al., 2009; Choy et al., 2014; Kumar et al., 2016). These motifs can be combined to tune the function and affinity of PP1 interactors as seen, for instance, with RepoMan and phosphatase 1 nuclear targeting subunits (PNUTS) that combine an RVxF motif, a ΦΦ motif, and an arginine residue to bind PP1 (Choy et al., 2014; Qian et al., 2015). In the crowded environment of the cell, these additional PP1 interaction motifs are important for controlling which holoenzymes are formed. Although the different isoforms of PP1 largely differ in their C-terminus and thus are all predicted to bind to the different PP1 binding motifs, recent elegant work has shown how subtle differences between PP1α and PP1γ can result in selective binding of PP1γ to RepoMan and Ki67 (Kumar et al., 2016).

The insight gained from analyzing PP1 holoenzymes has uncovered an unexpected level of complexity in their assembly and architecture. However, what is still lacking is a thorough understanding of what specific phosphorylation sites are targeted by specific PP1 holoenzymes in cells.

Substrate recognition by PP2A holoenzymes

In contrast to PP1, it has until recently been more enigmatic how PP2A holoenzymes recognize substrates. It is now clear that a conserved pocket on the B56 regulatory subunit binds to a SLiM, referred to as the LxxIxE motif present in multiple PP2A-B56 interactors (Hertz et al., 2016; Wang et al., 2016a,b; Wu et al., 2017). The LxxIxE motif was originally identified in the BubR1 checkpoint protein and subsequently in the protein RepoMan, providing the means to identify the motif in additional PP2A-B56 interactors (Suijkerbuijk et al., 2012; Kruse et al., 2013; Qian et al., 2013; Xu et al., 2013). In contrast to PP1-binding motifs in which phosphorylation blocks binding, for LxxIxE motifs phosphorylations within and downstream from the motif can rather increase PP2A-B56 binding, for instance, for controlling local interactions (Hertz et al., 2016). As an example, the interaction between the checkpoint protein BubR1 and PP2A-B56 is restricted to kinetochores because the BubR1 LxxIxE motif is only phosphorylated at kinetochores (Elowe et al., 2007, 2010; Huang et al., 2008; Kruse et al., 2013). Similarly, Aurora B and Plk1 likely control the association between RacGAP1 (Cyk4) and PP2A-B56 by phosphorylating the LxxIxE motif of RacGAP1 (Burkard et al., 2009; Hertz et al., 2016). While the LxxIxE motif binds to a conserved binding pocket present in all B56 isoforms, different isoforms display distinct localization patterns. For instance, B56γ and B56δ preferentially localize to kinetochores while B56γ and B56ε preferentially localize to the midzone during mitotic exit (Bastos et al., 2014; Nijenhuis et al., 2014). This localization is mediated by binding the LxxIxE motifs in BubR1 at kinetochores and Kif4A at the central spindle; however, it is presently unclear why only a subset of isoforms localizes to these structures. A possibility is that B56 isoform-specific contacts are present that further increase the affinity for BubR1 or Kif4A, leading to the preferential enrichment of isoforms.

Other important interactors of PP2A-B56 during mitosis are the Shugoshin proteins (SgoI and Sgo2) that protect centromeric cohesin through the recruitment of the phosphatase and might also affect kinetochore phosphorylations (Kitajima et al., 2006; Tang et al., 2006; Meppelink et al., 2015). However, SgoI does not contain an LxxIxE motif, binds a distinct region of B56, and contacts the catalytic subunit (Xu et al., 2009). The structure of the SgoI-PP2A-B56 complex has been determined by using a fragment of SgoI that has reduced binding affinity. Therefore, it is important that future work determines the structure of SgoI-PP2A-B56 containing the full binding domain of SgoI. It is puzzling that Sgo1 and Sgo2 also bind the protein SET, which is an inhibitor of PP2A and a histone chaperone (Li et al., 1996; Kitajima et al., 2006; Chambon et al., 2013). Why the Shugoshin proteins bind both PP2A-B56 and an inhibitor of this complex is unclear, but SET appears to also regulate the removal of Shugoshin proteins at later stages of mitosis (Krishnan et al., 2017).

The motif contributing to PP2A-B55 selectivity was discerned through a number of elegant mass spectrometry screens, which revealed that patches of basic residues upstream and downstream of SP or TP sites act as binding determinants of an acidic surface on the B55 regulatory subunit (Fig. 2 C; Cundell et al., 2016). Although direct binding between basic patches and PP2A-B55 still has to be demonstrated, the observations are consistent with the interaction between the Tau protein and SAMHD1 with PP2A-B55 (Xu et al., 2008; Schott et al., 2018). Several of the basic patches identified in PP2A-B55 substrates correspond to nuclear localization sequences in the targets. It is interesting to note that importin β has been proposed to regulate mitotic exit and bind to PP2A-B55 holoenzymes, raising the possibility that importin β can directly or indirectly regulate dephosphorylation of PP2A-B55 substrates (Schmitz et al., 2010). Importantly, the number of basic residues controls PP2A-B55 dephosphorylation kinetics, thus providing a mechanism for achieving temporal dephosphorylation of Cdk1 sites during mitotic exit and, thereby, coordination of mitotic exit events. This is in line with how temporal dephosphorylation by Cdc14, the budding yeast mitotic exit phosphatase, is guided by differences in catalytic efficiency among its substrates that is, in part, controlled by differences in

binding affinities to substrates (Bouchoux and Uhlmann, 2011). It is thus possible that a general principle controlling temporal dephosphorylation of mitotic exit substrates is the affinity of the phosphatases for substrates. Furthermore, meticulous reconstitution experiments with Cdc14 substrates have revealed that substrates with high catalytic efficiency delay the dephosphorylation of substrates with lower catalytic efficiency due to competition (Bouchoux and Uhlmann, 2011). Therefore, it is important that future work encompasses similar in vitro reconstitution experiments with PP2A complexes to investigate how dephosphorylation kinetics is affected by competition. Although substrate affinity is an important parameter, the amino acid composition of and surrounding the phosphorylation site is also important for controlling dephosphorylation kinetics. PP2A-B55 has a strong preference for phosphothreonine, and this orchestrates mitotic exit events (Cundell et al., 2016; Hein et al., 2017). Furthermore, the dephosphorylation kinetics of SP and TP sites is affected by the +2 position. A small, nonpolar amino acid in position +2 (S/TP-Gly sites) favors dephosphorylation while a proline in +2 (S/TP-Pro sites) hinders dephosphorylation, possibly due to restricted flexibility (McCloy et al., 2015).

Although our understanding of how PP2A-B56 and PP2A-B55 recognize their substrates has dramatically increased, it is very likely that further motifs in addition to the LxxIxE motif and basic patches contribute to recognition, as observed for PP1. For instance, additional contacts to the B subunits, scaffold subunit or catalytic subunit are all possible. In line with this idea, the sequence in the Eya1-4 proteins mediating binding to PP2A-B55 is very distinct from a basic patch (Zhang et al., 2018). Therefore, defining these putative motifs and understanding their role in mitotic regulation are important future goals.

PP6 regulation of mitosis

PP6 has been shown to regulate mitotic progression in yeast, flies, and human cells (Shimanuki et al., 1993; Bastians and Ponstingl, 1996; Goshima et al., 2003; Chen et al., 2007; Zeng et al., 2010). For instance, PP6 complexes control Aurora A activity through T-loop dephosphorylation as well as regulating components of the condensin I complex by removing CK2 phosphorylations (Zeng et al., 2010; Hammond et al., 2013; Rusin et al., 2015). Indeed, phosphoproteomic studies suggest that PP6 complexes act to counteract multiple CK2 sites during mitosis (Rusin et al., 2017). Currently, our understanding of how PP6 complexes recognize substrates is limited and, in principle, both the PPP6R subunits and ANR subunits could contribute to substrate selection. Because the PPP6R and ANR subunits contain folded domains, it is possible that they recognize SLiMs in substrates and regulators; however, this notion awaits validation. Alternatively, the unstructured region of PPP6R subunits could potentially bind to globular domains of substrates, as seen with Cdc25A, which uses the RxL motif to bind cyclins (Saha et al., 1997). The findings of a recent study possibly point in this direction in that the targeting of Plk1 to the PP6-ANR28-PPP6R2 complex occurs through phosphorylation of the unstructured region of PPP6R2, thereby creating a binding site for the polo-box domain of Plk1 (Kettenbach et al., 2018). The recruitment of Plk1 to PP6-ANKR28-PP6R2 seems to negatively regulate the complex, thus ensuring high levels of Aurora A activity during mitosis through suppression of Aurora A T-loop dephosphorylation.

Because PP6 is the least understood mitotic phosphatase, a fuller understanding of both the structural organization of the complex and its substrate recognition principles is an important goal for the future.

Regulation of mitotic phosphatase activities

Having described some of the basic principles of substrate recognition by protein phosphatases, I will now focus on the regulation of their activity because this is critical for proper cell division.

The regulation of inhibitory phosphorylations on Cdk1 controlled by Wee1/Myt1 kinases and Cdc25 phosphatases has been a fundamental model for describing entry into mitosis (Boutros et al., 2006; Lindqvist et al., 2009). It is now evident that, in addition to activating Cdk1, it is important to inhibit PP2A-B55, which appears to be a major Cdk1-antagonizing phosphatase (Agostinis et al., 1992; Mayer-Jaekel et al., 1993; Castilho et al., 2009; Mochida et al., 2009, 2010; Vigneron et al., 2009; Gharbi-Ayachi et al., 2010; Schmitz et al., 2010; Cundell et al., 2016). The pathway leading to PP2A-B55 inhibition has been extensively characterized and initiates with Cdk1 activation of the Mastl (Greatwall) kinase through phosphorylation of Cdk1 sites in Mastl (Fig. 3; Vigneron et al., 2011; Blake-Hodek et al., 2012). Upon Cdk1 phosphorylation, Mastl autophosphorylates, resulting in activation of the kinase. The relevant targets of Mastl are two small proteins, ENSA and Arpp19, that when phosphorylated by Mastl are transformed into potent inhibitors of PP2A-B55 (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). ENSA and Arpp19 share a short common Mastl phosphorylation motif, FDSGDY, that when phosphorylated inhibits PP2A-B55 with the phosphorylated residue binding to the active site of PP2A-B55 (Mochida, 2014). Interestingly, ENSA and Arpp19 inhibit PP2A-B55 by acting as substrates that are slowly dephosphorylated, and thus, when Mastl activity is turned off, PP2A-B55 activates itself by dephosphorylating ENSA and Arpp19 (Williams et al., 2014). Given that ENSA and Arpp19 are present at only roughly fivefold higher levels than PP2A-B55, activation of this phosphatase occurs in approximately 1 min after Mastl inactivation, ensuring rapid mitotic exit (Williams et al., 2014). This model has been termed "inhibition by unfair competition," and a similar mechanism has been shown to control the activity of the PP1-Mypt1 complex by the small protein inhibitor CPI-17 and could potentially be a general mechanism for controlling phosphatase activity (Filter et al., 2017). A small protein termed Bod1 has been proposed to be an inhibitor of PP2A-B56, and Bod1 is also phosphorylated to inhibit PP2A-B56; however, whether Bod1 inhibits through unfair competition is presently unclear (Porter et al., 2013). It should also be noted that Cdk1 might directly inhibit PP2A complexes through phosphorylation of a TP site in the C-terminal region of the catalytic domain, although the role of this in mitotic regulation has yet to be investigated (Evans and Hemmings, 2000; Longin et al., 2007; Kettenbach et al., 2011).

Activating phosphatases to promote mitotic exit

Anaphase marks the point of no return because the cells commit to mitotic exit, and in this and the following section, I will focus

Mitotic phosphatases in disease

The mitotic phosphatases generally act as tumor suppressors through dephosphorylation of substrates of oncogenic kinases. In most instances, it is unclear if the role of the phosphatases in mitosis plays a role in disease progression. PP2A was identified as the target of the small tumor antigen of the transforming viruses SV40 and polyomavirus, and small tumor antigen appears to mainly displace B56γ from the PP2A holoenzyme (Chen et al., 2004; Mumby, 2007). In addition, loss-of-function mutations in the PP2A scaffolding subunits as well as B56 regulatory subunits have been identified in a number of cancers and linked to intellectual disability and developmental disorders (Chen et al., 2005; Sablina et al., 2007; Nobumori et al., 2012; Houge et al., 2015; Haesen et al., 2016). The B55α subunit is down-regulated in prostate cancer, and PP2A-B55 might also affect the progression of Alzheimer's disease through dephosphorylation of the Tau protein (Gong et al., 1995; Cheng et al., 2011; Mao et al., 2011). An additional mechanism of PP2A inhibition in cancers is through the overexpression of CIP2A and SET, which are inhibitors of the phosphatase (Junttila et al., 2007; Zhou et al., 2017). PP2A-B56 also acts as a host factor for the Ebola virus while the HIV virus down-regulates PP2A-B56 (Greenwood et al., 2016; Kruse et al., 2018). Given the central role of PP2A in several human diseases, the development of PP2A modulators that either increase or decrease its activity is being developed (Lai et al., 2018; McClinch et al., 2018). PP6C is mutated in melanomas, and these mutations prevent the assembly of PP6 holoenzymes and hereby inhibit the phosphatase (Hodis et al., 2012; Krauthammer et al., 2012; Hammond et al., 2013). The PP6C mutations identified in cancers have been shown to cause chromosome missegregation because of increased Aurora A activity, and there is, thus, a link between PP6C mutations and their role in mitosis (Hammond et al., 2013). In addition, the PP6 holoenzymes have been identified as host factors for the influenza A virus (York et al., 2014). Disease mutations in PP1β have been linked to intellectual disabilities and delayed development, but if this is through an effect on mitosis is not clear (Hamdan et al., 2014; Ma et al., 2016a).

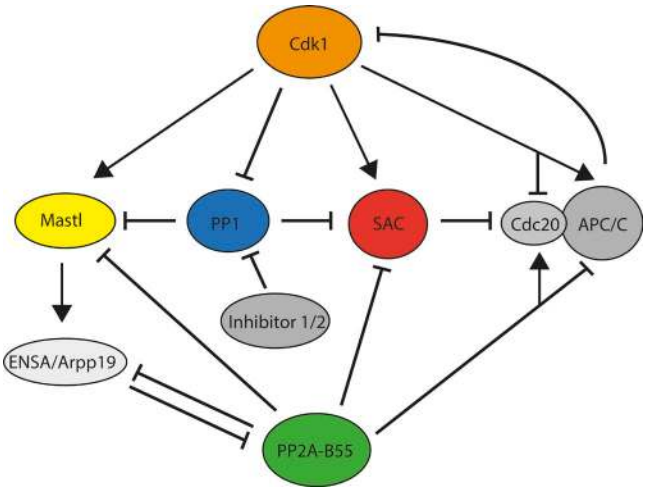

Figure 3. **Regulation of Cdk1 and PP2A-B55 activity.** A schematic of how the mitotic kinases and mitotic phosphatases antagonize each other during mitosis. Upon activation of APC/C-Cdc20, the activity of Cdk1 drops and results in activation of PP2A-B55 and mitotic exit. Arrows indicate stimulation of activity while lines with perpendicular lines indicate inhibition.

on the role and regulation of mitotic phosphatases in controlling mitotic exit events. This will illustrate the complex cross-talk between phosphatases and kinases and how regulated phosphatase binding helps coordinate mitotic events. Based on the "inhibition by unfair competition" model, the key event for activating PP2A-B55 is inactivation of Mastl through dephosphorylation. Removal of Cdk1 sites on Mastl is initiated by PP1, and then once PP2A-B55 is activated, it can also dephosphorylate Mastl (Heim et al., 2015; Ma et al., 2016b; Rogers et al., 2016; Ren et al., 2017). There is some disagreement on which Mastl phosphorylation sites are dephosphorylated by PP1, and it is also unclear if a specific PP1 holoenzyme is responsible because multiple PP1 regulatory subunits have been identified in Mastl purifications (Rogers et al., 2016; Ren et al., 2017). The Fcp1 phosphatase has also been implicated in dephosphorylation of ENSA and Mastl, but given the essential role of Fcp1 in dephosphorylating the RNA polymerase C-terminal domain, these data are difficult to interpret (Visconti et al., 2012; Hégarat et al., 2014; Williams et al., 2014). Furthermore, studies of fission yeast suggest that PP1 directly binds PP2A-B55 through an RVxF motif in B55 to activate PP2A-B55, and this mechanism might also extend to humans because the binding site for PP1 in B55 is conserved (Grallert et al., 2015). In fission yeast, the activated PP2A-B55 dephosphorylates B56 subunits to allow binding of PP1 and activation of PP2A-B56 (Grallert et al., 2015).

What initiates PP1-mediated dephosphorylation of Mastl? One mechanistic proposal is that PP1 activity is directly inhibited through the cyclin B1-Cdk1 phosphorylation of a C-terminal phosphorylation site (Thr320 in PP1γ). Indeed, phosphomimetic substitution of Thr320 inactivates PP1 and inhibits mitotic exit (Dohadwala et al., 1994; Kwon et al., 1997; Wu et al., 2009; Grallert et al., 2015). At metaphase, when the APC/C-Cdc20 complex is activated and initiates cyclin B1 degradation and thereby Cdk1 inactivation, PP1 autodephosphorylates, leading to its activation (Wu et al., 2009). Modeling suggests that Cdk1 activity has to be reduced by 90% before PP1 gets activated; however, this seems inconsistent with how fast PP2A-B55 is activated and the reported rates of cyclin B1 degradation (Clute and Pines, 1999; Cundell et al., 2013; Rogers et al., 2016). This inconsistency is possibly explained by the fact that the stoichiometry of Thr320 phosphorylation is 60% in prometaphase-arrested cells, which would not be sufficient to fully inhibit PP1 (Olsen et al., 2010). Consistent with PP1 being active in prometaphase is the observation that the mutation of the RVxF motif in Knl1 leads to an increased phosphorylation of Knl1 MELT repeats that are targeted by PP1 (Nijenhuis et al., 2014; Zhang et al., 2014). Second, the addition of PP1 T320A, which cannot be inhibited by Cdk1, to a *Xenopus laevis* extract only promotes mitotic exit at protein levels 8 times higher than endogenous PP1 while lower levels of PP1 T320A have no effect (Wu et al., 2009). A search for additional PP1 inhibitory activities that control PP1 during mitosis identified inhibitor 1 (PPP1R1A, expressed only in vertebrates) as this activity. Inhibitor 1, when phosphorylated by PKA, inhibits PP1, and similar to ENSA/Arpp19, PP1 dephosphorylates inhibitor 1 to release PP1 from inhibition (Wu et al., 2009). The combined action of PP1 Thr320 phosphorylation and inhibitor 1 is likely to be important for constraining PP1 activity. However, the picture is even more complicated because inhibitor 2 regulates PP1 mitotic activity and is possibly regulated by Cdk1 phosphorylation (Villa-Moruzzi, 1992; Puntoni and Villa-Moruzzi, 1995; Tung et al., 1995; Wang et al., 2008).

Although the complexity of the mechanisms regulating mitotic exit is beginning to unfold, there are currently many unknown parameters that need to be determined to fully understand how mitotic exit is regulated. How does PP1 activity change

both temporally and spatially during mitosis? What are the PP1 complexes that coordinate Mastl dephosphorylation to promote exit? Is Mastl activity locally controlled—as indicated by immunofluorescence analysis with a phosphospecific antibody recognizing a Cdk1-activating phosphorylation in Mastl (Hégarat et al., 2014)—and, if so, how? Furthermore, a temporal and quantitative description of all important phosphorylation sites and their stoichiometry, as well as the kinases and phosphatases involved, is needed to gain a proper understanding and modeling of mitotic exit. This could possibly be achieved by mass spectrometry, although this method lacks spatial information that has often transpired to be critical in the regulation of mitosis.

Regulation of APC/C-Cdc20 activity by phosphatases

The activity of APC/C-Cdc20 is tightly controlled because this complex is responsible for degrading cyclin B1 and thereby promoting mitotic exit at two levels: turning off Cdk1 and activating PP2A-B55 indirectly through turning off Mastl. Phosphatases regulate APC/C activity at two levels: directly through dephosphorylation of Cdc20 and APC/C subunits and indirectly through phosphatase-mediated silencing of checkpoint signaling from the kinetochores.

Improperly attached kinetochores activate the SAC to inhibit APC/C-Cdc20 activity, which ensures proper biorientation of chromosomes before anaphase is initiated (Lara-Gonzalez et al., 2012). The recruitment of Mps1 kinase to kinetochores initiates a phosphorylation cascade, including the MELT repeats in the Knl1 kinetochore protein, phosphorylation sites in the Bub1 checkpoint protein to facilitate Mad1 binding, and phosphorylation of Mad1 leading to its activation (Fig. 4; London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012; Faesen et al., 2017; Ji et al., 2017; Zhang et al., 2017). It has been suggested that Mps1 is activated by Cdk1 phosphorylation and inactivated by PP2A-B55 dephosphorylation, although different Mps1 phosphorylation sites were studied (Morin et al., 2012; Diril et al., 2016). This explains the dependency of the checkpoint on Cdk1 activity, although Mps1 is likely not the only target of Cdk1 in the checkpoint (Vázquez-Novelle et al., 2014). The MELT repeats in Knl1 act as binding sites for Bub1-Bub3 and BubR1-Bub3, and this indirectly results in the recruitment of PP2A-B56 through direct binding of the phosphatase to BubR1 (Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013). This kinetochore-localized pool of PP2A-B56 counteracts Aurora B activity to facilitate kinetochore-microtubule interactions through dephosphorylation of kinetochore proteins and negatively regulates checkpoint signaling by dephosphorylating Bub1 to prevent its binding to Mad1 and the RVxF motif in Knl1 to promote PP1 binding (Foley et al., 2011; Suijkerbuijk et al., 2012; Nijenhuis et al., 2014; Qian et al., 2017). PP2A-B56–mediated dephosphorylation of Bub1 has been proposed to act as a timer in the checkpoint, thereby restricting the Bub1-Mad1 interaction to a limited window in the early stages of mitosis. This timer is established by a delay in the recruitment of PP2A-B56 to kinetochores compared with Bub1 and Mps1 (Qian et al., 2017). The dephosphorylation of the RVxF motif in Knl1 results in the recruitment of PP1 and the dephosphorylation of MELT motifs, thereby preventing the binding of Bub proteins and turning off the checkpoint (Meadows et al., 2011; Rosenberg et al., 2011; London et al., 2012; Nijenhuis et al., 2014; Zhang et al., 2014). It is possible that PP2A-B56 bound to BubR1 can also dephosphorylate MELT repeats, and it might be that PP1 and PP2A-B56 act somewhat redundantly in dephosphorylating Knl1 (Espert et al., 2014). Such a redundancy could explain the minor delays in the checkpoint silencing observed in cells expressing Knl1 with a mutated RVxF motif or BubR1 with a mutated LxxIxE motif (Espeut et al., 2012; Espert et al., 2014; Nijenhuis et al., 2014; Zhang et al., 2014). Alternatively, PP1 and PP2A-B56 might act on multiple substrates to turn off checkpoint signaling, and simply preventing the dephosphorylation of a subset of substrates is insufficient to strongly impair checkpoint silencing. Furthermore, additional kinetochore interactors for PP1 exist, such as Mypt1, ELYS, Kif18A, CENP-E, and the Ska complex; however, the exact contribution of these PP1 interactors to SAC silencing is unclear (Yamashiro et al., 2008; Kim et al., 2010; Matsumura et al., 2011; Meadows et al., 2011; Häfner et al., 2014; Hattersley et al., 2016; Sivakumar et al., 2016). Understanding how closely localized kinetochore phosphatases precisely select the residues to be dephosphorylated in a temporal, controlled manner is clearly an important but challenging topic.

When the SAC signal is turned off at kinetochores, APC/C-Cdc20 becomes active, and this requires selective dephosphorylation of Cdc20 (Labit et al., 2012; Craney et al., 2016; Hein et al., 2017; Kim et al., 2017; Lee et al., 2017). Cdc20 is inhibited by phosphorylation on multiple sites by Cdk1 and Bub1, and these have to be removed while still maintaining activating Cdk1 phosphorylations on APC/C (Fujimitsu et al., 2016; Qiao et al., 2016; Zhang et al., 2016). This selective dephosphorylation of Cdc20 can at least, in part, be attributed to the fact that Cdk1 inhibitory sites in Cdc20 are TP while activating Cdk1 sites in APC/C are SP, resulting in selective Cdc20 dephosphorylation by PP2A-B55 due to its inherent preference for phosphothreonine (Hein et al., 2017). However, PP2A-B55 cannot be the only phosphatase for Cdc20 because APC/C-Cdc20 must be activated before PP2A-B55 to initiate Mastl inactivation. The identity of this phosphatase awaits discovery, but work in *Xenopus* suggests that it might be a PP2A complex and, indeed, PP2A-B56 has been shown to interact with APC/C in early mitosis (Labit et al., 2012; Craney et al., 2016; Lee et al., 2017). Furthermore, PP1 has been implicated in controlling Cdc20 dephosphorylation in worms (Kim et al., 2017). Understanding mitotic exit will require a full understanding of how different phosphatases regulate APC/C-Cdc20.

Major obstacles and possible solutions

While the central role of PPPs in regulating mitosis has been recognized for decades, it is only recently that the complexity of their regulation and targeting has started to unfold. However, a major impediment still remaining is our limited understanding of the precise substrates of the different PPP holoenzymes due to the absence of tools to precisely inhibit these complexes. This prevents the system-wide substrate identification that has been achieved for mitotic kinases. One solution is to generate more selective inhibitors for PPPs and specific holoenzymes, and progress has indeed been made in this direction (Fontanillo et al., 2016; Choy et al., 2017; Krzyzosiak et al., 2018). Alternatively,

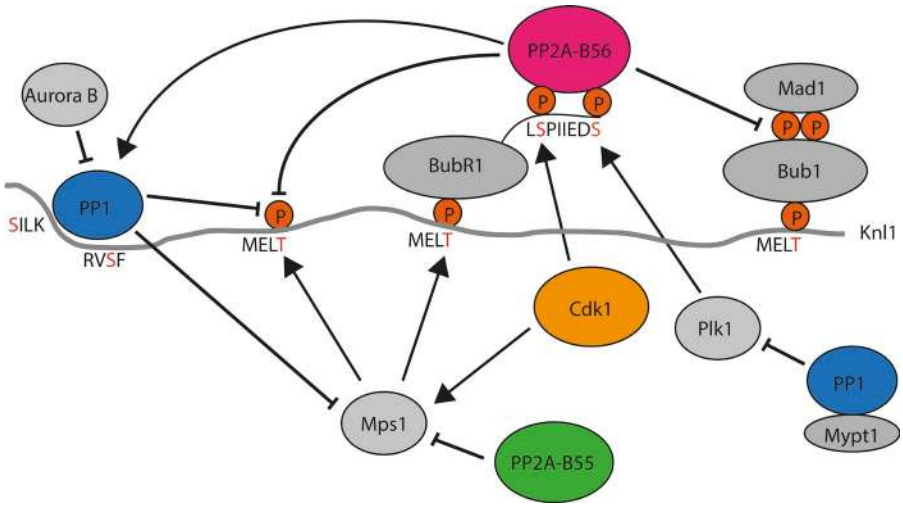

Figure 4. **Regulation of checkpoint activity at kinetochores by mitotic phosphatases.** The outer kinetochore protein Knl1 acts as a scaffold for coordinating the SAC signal, and this is regulated by kinases and phosphatases at multiple levels. Aurora B phosphorylates the PP1-binding site in Knl1, which prevents PP1 binding while PP2A-B56 bound to BubR1 stimulates PP1 binding through dephosphorylation of the PP1-binding site. It is likely that regulation of Mps1 by PP2A-B55 does not occur at kinetochores but is restricted to mitotic exit, whereas all other phosphorylation and dephosphorylation events are localized to kinetochores.

as our understanding of substrate recognition increases, it might be possible to target the phosphatase-SLiM interactions because they are low micromolar affinity interactions. Indeed, the immunosuppressants FK506 and cyclosporin A target the SLiM-binding pocket of Calcineurin, confirming that this is a potential strategy (Grigoriu et al., 2013). Establishment of the "phosphatome" for the different mitotic phosphatases and potentially specific holoenzymes would clearly allow for a better understanding of how these enzymes coordinate different mitotic events. However, such approaches would need to be complemented with meticulous in vitro assays to determine dephosphorylation kinetics and how this is influenced by the affinity, position, and nature of phosphorylation sites. From such systematic analyses, it might be possible to extract general principles that could be useful in interpreting the "phosphatome" data. Such combined information would not only provide an important overview but also help in the design of more precise experiments aimed at addressing the function of specific mitotic phosphatases.

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