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Spindle Checkpoint Silencing: PP1 Tips the Balance

Minireview

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The spindle checkpoint is a mitotic surveillance mechanism that delays anaphase until all sister chromatids are correctly attached to microtubules from opposite poles. Recent studies reveal that protein kinase Aurora B is a key regulator of spindle checkpoint activation whereas protein phosphatase PP1 antagonizes Aurora B and induces checkpoint silencing. Chromosome biorientation stretches the kinetochores and spatially separates centromeric Aurora B from its kinetochore substrates, comprising several PP1-interacting proteins (PIPs). The ensuing dephosphorylation of these PIPs creates docking sites for the bulk recruitment of PP1 to the kinetochores. We propose that this tension-induced targeting of PP1 triggers checkpoint silencing by the dephosphorylation of kinetochore and checkpoint components, including Aurora B substrates. In addition, PP1 also directly inactivates a kinetochore-associated pool of Aurora B and silences checkpoint signaling by opposing the centromeric targeting of Aurora B.

Introduction

During cell division both daughter cells receive one copy of each chromosome. Faithful chromosome segregation requires that sister chromatids be attached to microtubules from the opposite poles before their separation during anaphase. The spindle checkpoint has evolved as a eukaryotic surveillance mechanism that delays the onset of anaphase until all sister chromatids have achieved a bipolar attachment [1,2]. The key components of this checkpoint are the protein kinases Aurora B, Bub1, BubR1/Mad3 and Mps1, and the non-kinase proteins Bub3, Mad1 and Mad2. These checkpoint proteins collectively prevent the precocious activation of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that marks securin and cyclin B for proteolytic degradation. The removal of securin releases separase, which triggers anaphase by cleaving the protein (cohesin) that keeps the sister chromatids together at the centromeres. Cyclin B degradation inactivates protein kinase Cdk1 and promotes mitotic exit. Checkpoint signaling induced by un- or mal-attached sister chromatids generates protein complexes that bind and inhibit the APC/C activator Cdc20. The major spindle checkpoint effector is the mitotic checkpoint complex (MCC), consisting of BubR1, Bub3, Mad2 and Cdc20 (Figure 1).

The coupling between (in)correct spindle microtubule binding and checkpoint signaling occurs at the kinetochores, large protein complexes that are assembled on the centromeres. The inner kinetochore consists of CENP proteins, which are for the most part associated with the centromeres throughout the cell cycle and organize the underlying chromatin. In contrast, the proteins of the outer kinetochore are only assembled on the inner kinetochore at the beginning of mitosis. The core of the outer kinetochore is formed by the KNL1/Mis12/Ndc80 (KMN) protein complex (Figure 1). Ndc80 forms an attachment site for spindle microtubules but also probably binds the checkpoint protein Mps1 [3]. Likewise, KNL1 (also called CASC5 or Blinkin in vertebrates) interacts with spindle microtubules and the checkpoint kinases Bub1 and BubR1. Mad1 and Bub1 function as kinetochore scaffolds for the recruitment of other checkpoint proteins but may also interact with each other to induce the formation of the MCC and its subcomplexes [2].

Reversible protein phosphorylation has emerged as a key regulatory mechanism of the spindle checkpoint. Several recent studies identified protein kinase Aurora B, which is largely associated with the inner centromeres during (pro) metaphase, as a master regulator of spindle checkpoint signaling (Figure 1) [3–6]. Aurora B directly contributes to the recruitment of the checkpoint kinases Mps1, Bub1 and BubR1 to unattached kinetochores [3,6,7]. This results in supplementary phosphorylations (Table 1) [8–23], the recruitment of other checkpoint components, and the formation of mitotic checkpoint (sub)complexes [3,6,7]. In addition, Aurora B phosphorylates multiple subunits of the KMN network, which destabilizes erroneous kinetochore–microtubule interactions [10,20,24]. Once sister kinetochores achieve a bipolar attachment, the kinetochores are stretched by the pulling forces exerted by attached microtubules emanating from the opposite poles [14,25–28]. This spatially separates bulk centromeric Aurora B from its outer kinetochore substrates. The ensuing decreased phosphorylation of these substrates stabilizes microtubule–KMN interactions. When all kinetochores are bioriented and under tension the spindle checkpoint is satisfied and stops producing MCC or its inhibitory subcomplexes.

Protein Phosphatase PP1 Is an Essential Spindle Checkpoint Silencer

The spontaneous turnover of phosphoproteins and mitotic checkpoint (sub)complexes is too slow to account for the abrupt activation of the APC/C, hinting at the existence of switch-like, active processes that trigger checkpoint silencing [29]. Although numerous silencing mechanisms have been described (e.g., the competitive disruption of MCC-type complexes, and the covalent modification and inactivation of various checkpoint proteins by ubiquitination, deacetylation and proteolysis), these mechanisms are often not phylogenetically conserved or occur too late in mitosis to affect the onset of anaphase. Possibly, some of these mechanisms function in the local fine-tuning of mitotic checkpoint (sub)complexes. In contrast, the dephosphorylation of kinetochore and checkpoint proteins has recently come to the forefront as an essential and ubiquitous mechanism for spindle checkpoint silencing. Initial evidence came from observations that the expression of phospho-mimetic mutants of some KMN or spindle checkpoint proteins, or the inhibition of their dephosphorylation, activates the checkpoint constitutively, strongly suggesting that dephosphorylation

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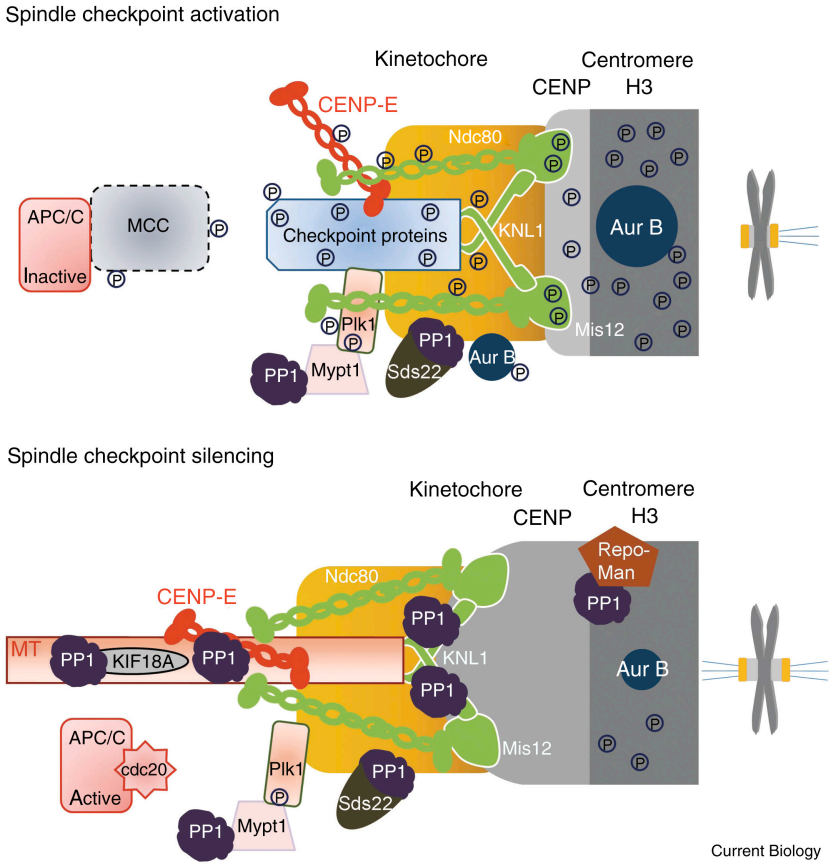

Figure 1. Phospho-regulation of the spindle checkpoint.

(A) Sister chromatids that are not properly bioriented are not under tension. This allows centromeric Aurora B (AurB) to phosphorylate outer kinetochore proteins, which destabilizes kinetochore-microtubule interactions and generates unattached kinetochores that can re-engage in microtubule binding. Aurora B signaling also contributes to the recruitment of spindle checkpoint components to free kinetochores and the production of the mitotic checkpoint complex (MCC). (B) When sister chromatids achieve a bipolar attachment and come under tension, Aurora B is partially removed from the centromeres. The remaining fraction of Aurora B gets spatially separated from its outer kinetochore substrates by stretching of the kinetochores. The ensuing reduced phosphorylation of Aurora B substrates promotes the recruitment of PP1 by KNL1, KIF18A (Klp5/6 in yeast) and CENP-E to the kinetochores. Other PP1 holoenzymes (PP1/Sds22, PP1/Mypt1 and PP1/Repo-Man) are targeted to the kinetochores or centromeres in a tension-independent manner. MT, microtubule.

at the kinetochores is an essential step in checkpoint silencing [12,30,31]. Consistent with this notion, the functional disruption of the kinetochore-associated protein phosphatase PP1 causes a metaphase arrest [32]. It has been challenging to determine whether this arrest stems from a checkpoint silencing defect, as manifested by a delayed anaphase in the presence of a perfectly aligned metaphase plate, and/or spindle–kinetochore interaction defects causing checkpoint activation. However, elegantly designed assays in yeast eventually made it possible to distinguish between these possibilities. Vanoosthuyse and Hardwick [33] demonstrated in fission yeast that checkpoint silencing requires PP1, independent of its role in kinetochore–microtubule stabilization. A silencing defect was detected after loss of the kinetochore-localized isoform of PP1 (dis2), but not after a lack of a distinct PP1 isoform (Sds21) or other mitotic phosphatases (PP2A and Cdc14), attesting to the specificity of kinetochore-associated PP1 in silencing the checkpoint. Pinsky *et al.* [34] independently reported that budding yeast becomes hypersensitive to checkpoint activation after expression of an inactive PP1 mutant, whereas overexpression of PP1 prevents checkpoint activation in response to both tension and attachment defects. Collectively, these data provide direct and strong evidence for a role of kinetochore-associated PP1 in checkpoint silencing.

The Diversity of PP1 Kinetochore-Anchoring Proteins

The purified catalytic subunit of PP1 has a rather broad substrate specificity [35]. However, in the cell PP1 is associated with numerous PP1-interacting proteins (PIPs) that determine precisely when and where the phosphatase acts. PIPs target PP1 to specific subcellular compartments (e.g., nucleoli, centrosomes, the mitotic spindle and chromosomes) that contain subsets of substrates. They also often act as substrate specifiers by providing additional docking sites for selected substrates and preventing the recruitment of others. Sometimes, PIPs themselves are substrates for PP1. Generally, they compete with each other for binding to PP1 via multiple, short (4–8 residues) and degenerate docking motifs. Best characterized are the so-called 'RVxF' and 'SILK' motifs. The RVxF motif mediates PP1 binding of approximately 70% of the ~200 known vertebrate PIPs. Six of these RVxF-containing PIPs also have a SILK motif.

Several kinetochore-associated PIPs have been identified (Figure 1). KNL1, also known as Spc7 in fission yeast and Spc105 in budding yeast, is thus far the only PIP shown to be essential for the bulk recruitment of PP1 to the kinetochores in metaphase [15,17,32]. It has both RVxF- and SILK-type PP1 docking motifs, but their relative contribution to PP1 anchoring is species dependent [15,32]. A PP1-binding mutant of KNL1 is correctly targeted to the kinetochores, but cells expressing this mutant show a deficient dephosphorylation of kinetochore-associated Aurora B substrates and a persistent activation of the spindle checkpoint [15,17]. Forced targeting of PP1 to the kinetochores via fusion to the kinetochore protein CENP-B or to a PP1-binding mutant of KNL1 can rescue this phenotype, indicating that KNL1 has a PP1-targeting but not a substrate-specifying function [17,32]. In contrast, a fusion of PP1 and wild-type KNL1, expected to target twice the normal amount of PP1 to the kinetochores, is lethal, demonstrating that the level of PP1 that is needed at the kinetochores must be precisely titrated [17]. The recruitment of PP1 by KNL1 in human cells is regulated by tension at the

Table 1. Candidate substrates of PP1 in spindle checkpoint signaling.

Checkpoint protein	Phosphorylated residues	Kinases identified	Organism	Function of phosphorylation	Refs
Aurora B	T232	Aurora B	*H. sapiens*	Kinase activation	[16]
Mps1	T12, S15	Mps1	*H. sapiens*	Kinetochore localization	[21]
	T676	Mps1	*H. sapiens*	Kinase activation	[21]
	S844	MAPK	*X. laevis*	Kinetochore localization	[23]
Mad1	T680, T708	Plk1	*H. sapiens*	Kinetochore localization	[21]
Mad2	S195	ND	*H. sapiens*	Conformational inactivation; binding to MAD1	[21]
	S170, S178, S195	ND	*H. sapiens*	Binding to APC/C	[21]
BubR1	S670	Mps1	*H. sapiens*	Destabilization of MT binding; spindle checkpoint activation	[12] [12]
	S1043	Mps1	*H. sapiens*	Destabilization of MT binding	[21]
	T620	Cdk1	*H. sapiens*	Recruitment of Plk1	[21]
	S676	Plk1	*H. sapiens*	Stabilization of MT binding	[21]
	T792, T1008	Plk1	*H. sapiens*	Kinase activation	
Mad3p	S10, S303, S337, S486	Ipl1	*S. cerevisiae*	Spindle checkpoint activation	[13]
Bub1	ND	Bub1	*H. sapiens*	Kinase activation	[18]
	T482, T493, S500, S634, T650	MAPK	*X. laevis*	Spindle checkpoint activation	[50]
Bub1p	T340, T423, T455, S466	cdc2	*S. pombe*	Spindle checkpoint activation	[22]
	T566	cdc28	*S. cerevisiae*	Decreased protein stability	[11]
Cdc20	S41, S72, S92, S153, T157, S161	Bub1	*H. sapiens*	Spindle checkpoint activation	[21]
	T64, T68	MAPK	*X. laevis*	Spindle checkpoint activation	[9]
	S50, T79	ND	*X. laevis*	Spindle checkpoint activation	[9]
Ndc80/Hec1	S5, S15, S44, T49, S55, S69	Aurora B	*H. sapiens*	Destabilization of MT binding	[10]
	S165	Nek2	*H. sapiens*	Destabilization of MT binding; spindle checkpoint activation	[10,19,21]
KNL1	S24, S60	Aurora B	*H. sapiens*	Destabilization of MT binding; spindle checkpoint activation	[14,20] [15,17]

ND, not determined; MT, microtubule.
All listed sites were determined by site-specific methods. For phosphorylation sites that were only assigned by proteomic discovery-mode mass spectrometry or for which the physiological significance is not yet defined, see PhosphoSitePlus® (Cell signaling). The list is limited to sites that have an established function in spindle checkpoint signaling.

kinetochores [32]. This tension-dependency can at least partially be explained by the observation that Aurora B prevents binding of PP1 by phosphorylation of a serine residue within the RVxF docking motif [32]. Thus, Aurora B not only phosphorylates KMN and spindle checkpoint proteins but also prevents the recruitment of the opposing PP1. The formation of the PP1/KNL1 holoenzyme not only requires the inactivation of Aurora B and/or its tension-induced separation from kinetochore substrates but also the dephosphorylation of the PP1-docking site by a hitherto unidentified phosphatase (see also below).

Meadows *et al.* [15] recently identified the plus-end directed motor protein Klp5/6 (kinesin 8), which functions in both chromosome congression and biorientation, as an additional kinetochore-anchoring PIP in fission yeast. Recruitment of PP1 by both Klp5/6 and KNL1 is necessary to silence the spindle checkpoint in this model system. KIF18A, the vertebrate orthologue of Klp5/6, and CENP-E (kinesin-7) also interact with PP1 [15,31]. Moreover, the phosphorylation of CENP-E by Aurora B at the kinetochores decreases its affinity for microtubules and disrupts PP1 binding. Conversely, the dephosphorylation of a threonine residue in its RVxF-type docking motif enables the recruitment of PP1 and this is required for stable biorientation of chromosomes congressed by CENP-E.

A third ubiquitous kinetochore-targeting subunit of PP1 is Sds22 (Figure 1), one of the most ancient and conserved PIPs [16]. Sds22 and PP1 mutually depend on each other for kinetochore recruitment, suggesting that the interaction of Sds22 with the kinetochores depends on its prior association with PP1 [16]. Depletion experiments revealed that Sds22 is only associated with a minor fraction of kinetochore-associated PP1. PP1/Sds22 dephosphorylates and inactivates a small pool of Aurora B that is localized at the kinetochores. The function of this PP1/Sds22-regulated fraction of Aurora B is not clear but is likely to be different from that of the bulk of Aurora B in the centromeres since a depletion of Sds22 does not prevent the generation of interkinetochore tension and the associated dephosphorylation of the tested Aurora B substrates [16]. Possibly, the PP1/Sds22-regulated pool of kinetochore-associated Aurora B keeps the RVxF-type docking motifs of KNL1 and CENP-E phosphorylated during metaphase whereas other kinetochore substrates may be preferentially phosphorylated by centromeric Aurora B. Interestingly, PP1/Sds22 is already present at the kinetochores in prometaphase and is therefore an excellent candidate to trigger checkpoint silencing by dephosphorylating both the PP1 docking sites of KNL1 and CENP-E as well as the counteracting pool of Aurora B. It is currently unknown how the activity of PP1/Sds22 is regulated and whether it is also affected by tension between sister kinetochores. Sds22 does not have an RVxF-type PP1 docking motif but forms a trimeric complex with PP1 and the RvXF-containing Inhibitor-3, the orthologue of Ypi1 in budding yeast [35]. Since a loss of Inhibitor-3 results in a metaphase arrest due to the activation of the spindle checkpoint [36], it probably acts as a positive regulator of kinetochore-associated PP1/Sds22.

Yet another kinetochore-associated phosphatase is the metazoan PP1/Mypt1, which binds directly to protein kinase Plk1 and restrains its activity [37]. Since Plk1 phosphorylates several spindle checkpoint proteins (Table 1) [8–23] and promotes the acute recruitment of Aurora B to unattached

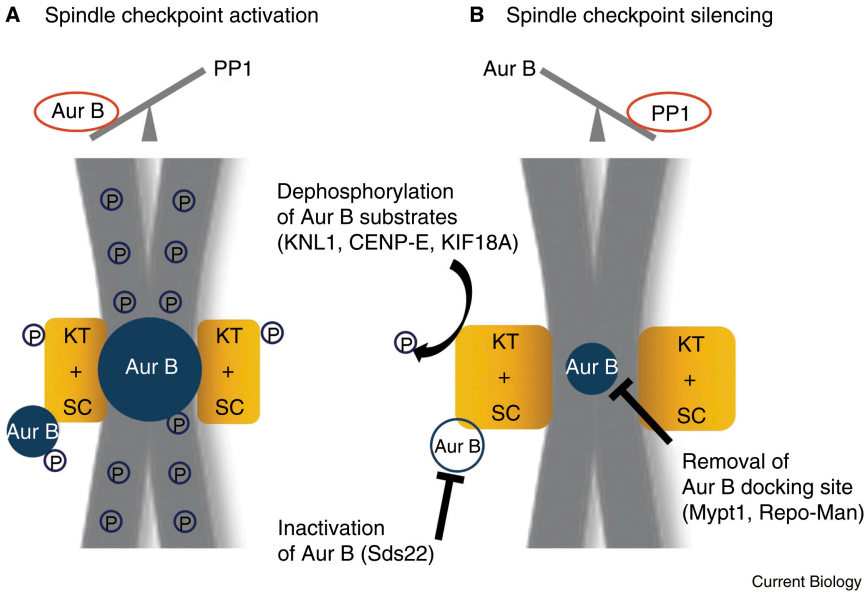

Figure 2. The balance of power between Aurora B and PP1 in checkpoint signaling.

(A) During (pro)metaphase Aurora B (Aur B) phosphorylates various kinetochore (KT) and spindle checkpoint (SC) proteins, resulting in the destabilization of incorrect kinetochore–microtubule attachments and spindle checkpoint activation. (B) When sister chromatids come under tension, kinetochore-associated PP1 holoenzymes reverse Aurora B signaling and trigger spindle checkpoint silencing. PP1 dephosphorylates Aurora B, Aurora B substrates and a centromeric Aurora B docking site. The involved PP1-interacting proteins (PIPs) are listed between brackets.

chromatids in diploid cells [38], the counteracting PP1/Mypt1 possibly contributes to the stabilization of spindle–kinetochore interactions and spindle checkpoint silencing (Figure 1). Repo-Man is a vertebrate-specific chromosome-associated PIP [39]. PP1/Repo-Man was recently shown to dephosphorylate histone H3 on Thr3 (H3T3) during mitosis [40,41]. This is relevant for spindle checkpoint signaling because phosphorylated H3T3 serves as a centromeric docking site for the Aurora B complex. The dephosphorylation of H3T3 on the chromosome arms prevents the spreading of Aurora B beyond the centromeres in (pro)metaphase, whereas the centromeric dephosphorylation of H3T3 in anaphase is likely to be one of the key steps in the translocation of Aurora B to the spindle midzone. This translocation is important because it prevents the re-initiation of spindle checkpoint signaling once tension between sister chromatids is lost at the onset of anaphase [7].

Finally, Fin1 is a yeast-specific kinetochore PIP that regulates checkpoint silencing in a PP1-dependent manner [42]. The dephosphorylation of Fin1 by PP1 removes associated 14-3-3 protein and increases its affinity for kinetochores. Yeast has a closed mitosis, implying that it must have mechanisms for the timely nuclear accumulation of PP1, needed for spindle checkpoint silencing and the mitotic exit. Since the nuclear transport of PP1 depends on PIPs with a nuclear localization signal, it comes as no surprise that several proteins have been identified that are essential for the nuclear accumulation of PP1 in yeast during mitosis, including Torc1, Sds22, Ypi1 and Cdc48/Shp1 [36,43]. Deletion of any of these proteins results in a mid-mitotic arrest but this does not necessarily imply that they are all directly involved in checkpoint signaling.

Substrates of Kinetochore-Associated PP1

Compelling genetic and biochemical evidence from various model organisms shows that PP1 and Aurora B act antagonistically because they have common substrates [16,43], including various KMN and checkpoint proteins (Table 1) [8–23]. However, the phosphorylation sites that are functionally important for checkpoint signaling have not yet been identified. Moreover, a detailed map of kinetochore-associated substrates of PP1 is still missing and it is not yet clear whether there is specificity with respect to the order of dephosphorylation of these substrates and the involved PP1 holoenzymes. In addition, PP1 probably also reverses signaling by other checkpoint kinases. For example, microtubule capture by CENP-E silences BubR1-dependent checkpoint signaling [44]. An enticing hypothesis is that CENP-E bound PP1 plays a role in the inactivation of BubR1. Along the same lines, Bub1 directly phosphorylates and inhibits the APC/C activator Cdc20 [18]. Since Bub1 directly binds to KNL1, it is an attractive substrate for inactivation by KNL1-associated PP1 during checkpoint silencing. Another kinetochore-associated substrate of PP1 is the minus-end directed motor protein dynein, which contributes to checkpoint silencing by transporting Mad1/Mad2 and other checkpoint proteins along the captured microtubules from the kinetochore to the spindle poles [45]. The poleward streaming of dynein is induced by PP1-catalyzed dephosphorylation and represents an additional mechanism to couple chromosome biorientation to checkpoint inactivation [45]. Finally, PP1 also dephosphorylates kinetochore-associated proteins that are not implicated in checkpoint silencing *per se*, but link checkpoint silencing to the start of anaphase. These include the microtubule depolymerase MCAK (kinesin-13), a potential substrate of kinetochore-associated PP1 that contributes to chromosome movement by driving microtubule depolymerization [46]. The yeast protein Dam1, which links the energy released by microtubule depolymerization to force generation, is an established PP1 substrate [43,47].

Conclusions

Kinetochores function as hubs for spindle checkpoint signaling by various protein kinases and phosphatases [1,2]. The checkpoint kinases are connected by multiple positive feedback loops, e.g. between Bub1 and Aurora B, and they form negative feedback loops to their opposing phosphatases, e.g. between Aurora B and PP1. This combination of positive and negative feedback loops, often in association with phosphorylation of their substrates on multiple sites, creates robust bistable phosphoswitches [48]. We suggest that a similar arrangement, including positive feedback loops between kinetochore-associated phosphatases and negative feedback loops between these

phosphatases and their opposing kinases, induces abrupt spindle checkpoint silencing once all sister chromatids are bioriented.

In vertebrates at least six distinct PP1 holoenzymes counteract Aurora B signaling at the kinetochore (Figure 2). These phosphatases act by opposing the recruitment (PP1-Mypt1) or retention (PP1/Repo-Man) of Aurora B at the inner centromeres, inactivating kinetochore-associated Aurora B (PP1/Sds22), and dephosphorylating Aurora B substrates (PP1/KNL1, PP1/CENP-E and PP1/KIF18A). We suggest that the tight balance between Aurora B and PP1 during checkpoint signaling is tipped in favour of PP1 when the kinetochores come under tension and that this triggers checkpoint silencing. Although conclusive evidence is still lacking, it seems likely that kinetochore-associated PP1 is also implicated in the downregulation of other spindle checkpoint kinases, including Bub1, BubR1 and Mps1. Finally, elegant heterokaryon experiments performed more than a decade ago revealed that spindle checkpoint silencing also generates diffusible checkpoint inhibitors [49]. The nature of these soluble inhibitors has never been elucidated but we speculate that they could be cytoplasmic or nucleoplasmic (in yeast) PP1 holoenzymes that act in concert with kinetochore-associated PP1 to trigger the onset of anaphase. Clearly, the diverse functions of PP1 in spindle checkpoint signaling are just beginning to come to the surface.

Acknowledgements

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