Overcoming inhibition in the spindle checkpoint

PERSPECTIVE

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Spindle checkpoint silencing is a critical step during mitosis that initiates chromosome segregation, yet surprisingly little is known about its mechanism. Protein phosphatase I (PP1) was shown recently to be a key player in this process, and in this issue of *Genes & Deverlopment*, Akiyoshi and colleagues (pp. 2887–2899) identify budding yeast Fin1p as a kinetochore-localized regulator of PP1 activity toward checkpoint targets. Here we review recent mechanistic insights and propose a working model for spindle checkpoint silencing.

The spindle checkpoint delays anaphase onset until all chromosomes have established biorientation. When the checkpoint is activated, unattached kinetochores catalyze the formation of Mad2–Cdc20 and the mitotic checkpoint complex (MCC: Mad2–Cdc20–BubR1–Bub3). The MCC blocks the activity of the anaphase-promoting complex (APC/C^{Cdc20}), the E3 ubiquitin ligase that targets key inhibitors of mitotic exit (securin and cyclin B) for degradation. Upon correct attachment of all kinetochores to microtubules, the checkpoint is satisfied and stops producing APC/C inhibitors. However, the rate of spontaneous dissociation of these inhibitors is low, and it is widely accepted that an active process, referred to here as spindle checkpoint silencing, must be triggered for APC/C^{Cdc20} to be activated and for anaphase to proceed. Checkpoint silencing has received much attention of late, and several mechanisms have been proposed. Their relative contribution to checkpoint silencing and how their actions are coordinated remain unclear. Here we discuss the challenges that checkpoint silencing poses, both to cells and to researchers, and identify major obstacles that need to be overcome to improve our understanding of these mechanisms.

Checkpoint activation produces a diffusible signal

The key aspects of spindle checkpoint activation can be summarized as follows (for recent extensive reviews see Musacchio and Salmon 2007; Ciliberto and Shah 2009). The checkpoint targets Cdc20, the early mitotic activator of the APC/C. Unattached kinetochores recruit a stable Mad1/Mad2 complex, which catalyzes the production of Mad2–Cdc20 complexes. This is believed to be the rate-limiting step in checkpoint activation (Simonetta et al. 2009) and, as such, is a likely target of checkpoint silencing mechanisms. This complex then binds Mad3/BubR1 and Bub3 to form the MCC, and can then associate with mitotic APC/C to form APC/C^{MCC} (Nilsson et al. 2008; Kulukian et al. 2009). This has two consequences: (1) reduction of the ubiquitin ligase activity of the resulting APC/C^{MCC} by >80% (Herzog et al. 2009), and (2) APC/C-dependent Cdc20 degradation (Pan and Chen 2004; Nilsson et al. 2008), both of which prevent polyubiquitination of securin and cyclin B. A single unattached kinetochore is sufficient to inhibit APC/C^{Cdc20} activity, showing that the checkpoint is an extremely sensitive and efficient signaling mechanism.

Interestingly, Mad3/BubR1 does not need to be enriched at kinetochores to be an APC/C inhibitor (Kulukian et al. 2009; Malureanu et al. 2009; Vanoosthuyse et al. 2009), showing that checkpoint-dependent inhibition of APC/C^{Cdc20} can occur in the cytoplasm. It is thought that the production of APC/C inhibitors at unattached kinetochores is amplified in the cytoplasm, so that the whole pool of APC/C^{Cdc20} is inhibited. To fully silence the checkpoint upon chromosome biorientation, cells must inhibit the production of Mad2–Cdc20 and overcome the cytoplasmic amplification of APC/C inhibitors. However, how this is achieved remains largely unclear.

Understanding these mechanisms is made more difficult by the fact that, in mitosis, there can be at least three APC/C "isoforms" in the cell: apo-APC/C (the core complex with no cofactor bound) (Herzog et al. 2009), APC/C^{Cdc20}, and APC/C^{MCC}. To date, there are no exhaustive data available that quantify the relative abundance of these different APC/C complexes, their respective localization in the cell, or their relative stability. Moreover, it is unclear whether the abundance, localization, or activity of these APC/C isoforms is regulated, but CDK and checkpoint kinase activity seem likely to have important roles to play in this regard.

How far from kinetochores do checkpoint activation/inactivation signals travel?

To address this issue, Rieder et al. (1997) fused cells to produce heterokaryons containing two separate mitotic

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spindles (spindles 1 and 2). Unattached kinetochores on spindle 1 did not prevent spindle 2 from initiating anaphase (Rieder et al. 1997), showing that APC/C inhibitors could not diffuse effectively from one spindle to the next. On the other hand, once spindle 2 had committed to anaphase, spindle 1 soon followed, despite still having unattached kinetochores. This suggested that the APC/C-activating signals (checkpoint silencing) could readily diffuse from one spindle to the next. Cyclin B is first degraded on chromosomes and spindle poles (Clute and Pines 1999; Huang and Raff 1999), suggesting that the APC/C is first activated near chromosomes and spindles upon chromosome biorientation. CDK activity is important to maintain a spindle checkpoint arrest (Li and Cai 1997; Kitazono et al. 2003), so, potentially, the localized degradation of Cyclin B and the resulting localized loss of CDK activity could precipitate spindle checkpoint inactivation. In summary, checkpoint-dependent APC/C inhibition is first overcome around chromosomes and spindle poles, and checkpoint silencing signals are then amplified to diffuse throughout the cytoplasm.

There is a caveat with this model. If checkpoint silencing signals were amplified and totally free to diffuse, then it would be unlikely that a single unattached kinetochore could block anaphase onset. Indeed, one would predict that the silencing signals sent by all correctly attached kinetochores would overwhelm the checkpoint-activating signal sent by the single unattached kinetochore. Alternatively, some mechanisms might limit the amplification/diffusion of checkpoint-inactivating signals. Consistent with this idea, Bub1 kinase activity is required to block anaphase onset in response to a few unattached kinetochores (low concentration of the microtubule-depolymerizing drug nocodazole), but not when all kinetochores are unattached (high concentration of nocodazole) (Chen 2004). Bub1 kinase activity might help generate a more potent MCC (Tang et al. 2004; Vanoosthuyse and Hardwick 2005); alternatively, it might limit the spread of checkpoint silencing signals.

What cues inactivate the checkpoint?

Checkpoint silencing is directly linked to the correct attachment of kinetochores to microtubules, and it is widely accepted that the signals inactivating the checkpoint emanate from centromeres. However, there is still controversy regarding what exactly inactivates the checkpoint. Some believe that tension across centromeres imposed by chromosome biorientation is the signal (Liu et al. 2009; Santaguida and Musacchio 2009). Others believe that tension across centromeres has little effect on checkpoint silencing; instead, microtubule occupancy per se, whether or not it produces tension across centromeres, is sufficient to silence the checkpoint (Maresca and Salmon 2009; Uchida et al. 2009; Yang et al. 2009). In the latter model, microtubule occupancy induces conformational changes in the kinetochore (Wan et al. 2009), which somehow silence the checkpoint. Whatever the initial trigger, surprisingly few proteins have been implicated as silencing factors.

Checkpoint silencing: a difficult problem to tackle

Identifying components specifically involved in checkpoint silencing is difficult for several reasons.

(1) Mutants unable to silence the checkpoint (through their failure to disassemble APC/C inhibitors) exhibit the same "arrest" phenotype as mutants that continuously activate the checkpoint because of kinetochore–microtubule attachment defects. Both classes of mutants exhibit a checkpoint-dependent metaphase delay. In theory, these two classes of mutants can be distinguished: A "silencing" mutant should establish a metaphase plate normally but fail to commit to anaphase, while an "attachment" mutant should fail to establish a normal metaphase plate. In practice, however, even if cells form a normal-looking metaphase plate, it is difficult to rule out that minor kinetochore–microtubule attachment defects remain, which the checkpoint, but not current techniques, can detect. For example, recent studies characterized the Ska3 kinetochore component in vertebrates. While four studies (Gaitanos et al. 2009; Raaijmakers et al. 2009; Theis et al. 2009; Welburn et al. 2009) concluded that Ska3 is required for stable kinetochore–microtubule interactions, a fifth study (Daum et al. 2009) concluded that Ska3 was dispensable to form a proper metaphase plate, but was required for checkpoint silencing. All studies agree that Ska3 is required to establish the mature kinetochore–microtubule attachments that will eventually satisfy the checkpoint, probably placing Ska3 upstream of the machinery that disassembles the anaphase inhibitors rather than as part of it.

(2) As activation and silencing of the checkpoint are intimately linked to the attachment of kinetochores to microtubules, it is likely that certain players will regulate both processes simultaneously. To specifically study the role of such components in checkpoint inactivation requires separation-of-function alleles, which can be difficult to generate. Recently, such a separation-of-function allele was described for the kinetochore component Ndc80 in budding yeast (Kemmler et al. 2009). Ndc80 is critical for kinetochore–microtubule attachments and kinetochore recruitment of the checkpoint components. Ndc80 is phosphorylated by the checkpoint kinase Mps1, and a phospho-mimic mutant fails to inactivate the checkpoint without perturbing kinetochore–microtubule interactions (Kemmler et al. 2009). This suggests that Mps1-dependent phosphorylation of Ndc80 contributes to checkpoint activation, and that these phospho-modifications need to be removed for the checkpoint to be inactivated.

(3) With several pathways regulating anaphase onset, their redundancy might mask silencing defects. Recent data indicate that separase, the enzyme that triggers anaphase onset by cleaving cohesin once the checkpoint has been satisfied, is also inhibited in a checkpoint-independent manner (Clift et al. 2009; Fang et al. 2009). Thus, mutants failing to commit to anaphase after chromosome biorientation could in theory be defective in this separase regulatory pathway, rather than being checkpoint silencing mutants.

(4) Because of the intimate link between checkpoint silencing and chromosome biorientation, it has been difficult to develop specific assays to study checkpoint inactivation. However, we recently developed such an assay in fission yeast, based on the artificial inactivation of the checkpoint in the absence of chromosome biorientation. In this assay, the checkpoint is not satisfied, as kinetochores remain unattached throughout, but is instead silenced through direct inhibition of Aurora kinase activity (Vanoosthuyse and Hardwick 2009). This allowed us to look for downstream factors required for APC/C^{Cdc20} activation upon Aurora inhibition. Using this assay, we showed that $PP1^{Dis2}$ phosphatase activity (but not other centromeric phosphatases) is critical for checkpoint silencing and APC/C^{Cdc20} activation (Vanoosthuyse and Hardwick 2009). However, it is important to remember that this assay is carried out in the absence of microtubules and cannot be used to study the contribution of the spindle in checkpoint silencing.

Silencing mechanisms that emanate from kinetochores

As mentioned above, signals inactivating the checkpoint emanate from kinetochores under tension. To date, two centromere-generated silencing mechanisms have been proposed that are particularly attractive because they are directly regulated by chromosome biorientation.

The first mechanism proposes that dynein-dependent kinetochore stripping of the checkpoint components Mad2 and BubR1 upon kinetochore–microtubule attachment inactivates the spindle checkpoint (Howell et al. 2001). This idea is popular because it is predicted that the more kinetochore–microtubule attachments are stable, the greater the flux of Mad2 and BubR1 away from kinetochores will be. As maximum stability of attachment is achieved upon chromosome biorientation (tension across centromeres upon biorientation stabilizes the attachment), dynein-dependent kinetochore stripping potentially directly links chromosome biorientation to checkpoint inactivation. However, this model makes the assumption that spindle-localized Mad2/BubR1 cannot generate APC/C^{Cdc20} inhibition. This assumption remains unproven, and is somewhat challenged by the idea that APC/C^{Cdc20} inhibition can be amplified at sites other than kinetochores (Essex et al. 2009; Kulukian et al. 2009; Malureanu et al. 2009; Vanoosthuyse et al. 2009). Anaphase onset can occur without depleting Mad2 from kinetochores (Canman et al. 2002) and, conversely, prolonged checkpoint activation can occur despite depletion of Mad2 from kinetochores (Chan et al. 2009). These observations demonstrate that dynein-dependent kinetochore stripping of Mad2 and BubR1 cannot be sufficient to silence the checkpoint.

We recently proposed an alternative silencing mechanism for the checkpoint, which also emanates from kinetochores and is directly regulated by chromosome biorientation. On centromeres lacking tension, Aurora B-dependent phosphorylation of kinetochore substrates contributes to checkpoint activation and microtubule destablization (for review, see Ruchaud et al. 2007). Tension across centromeres imposed by chromosome biorientation displaces Aurora B kinase from its kinetochore substrates (Liu et al. 2009). However, this is not sufficient to silence the checkpoint. In order to rapidly inactivate the checkpoint and activate APC/C, kinetochore-localized protein phosphatase I (PP1) phosphatase must reverse Aurora-dependent and Mps1-dependent phosphorylation events at kinetochores (Pinsky et al. 2009; Vanoosthuyse and Hardwick 2009). In fission yeast, this function of Aurora kinase in checkpoint activation and PP1 phosphatase in checkpoint silencing does not require microtubules (Vanoosthuyse and Hardwick 2009). This shows that their checkpoint function is distinct from their function in the regulation of kinetochore–microtubule attachments. The substrates of Aurora kinase and PP1 phosphatase in this process are still unknown, but likely candidates are checkpoint components, APC/C^{Cdc20}, or kinetochore components such as Ndc80 (Kemmler et al. 2009). In fission yeast, lack of Bub3p prevents MCC components from associating stably with unattached kinetochores (Vanoosthuyse et al. 2009; Windecker et al. 2009), yet fission yeast Bub3p is largely dispensable for MCC formation and APC/C inhibition (Vanoosthuyse et al. 2009). However, Bub3p is required for efficient silencing of the spindle checkpoint (Vanoosthuyse et al. 2009). Our interpretation of these data is that checkpoint components such as Mad3p, which is phosphorylated by Aurora in budding yeast (King et al. 2007a), need to be enriched on kinetochores for efficient inactivation by PP1 phosphatase.

Although it remains to be formally proven, it is possible that kinetochore-localized PP1 also counteracts the microtubule-destabilizing activity of Aurora B on kinetochores, most likely through reversal of Aurora B-dependent modification of MCAK and/or Ndc80 (Andrews et al. 2004; DeLuca et al. 2006). If this were true, kinetochore-localized PP1 phosphatase would regulate spindle checkpoint in two ways: (1) indirectly, by stabilizing kinetochore–microtubule attachments that produce tension and thereby satisfy the checkpoint, and (2) directly, by silencing the checkpoint. Note that kinetochore-localized PP1 phosphatase activity also potentiates kinetochore stripping of checkpoint components upon chromosome biorientation (Whyte et al. 2008), showing that PP1 probably contributes to checkpoint inactivation in several ways.

Toward the identification of regulators and substrates of PP1 phosphatase

How can one identify the substrates of PP1 that are important for spindle checkpoint silencing? The specificity of PP1 is ensured by tight regulation of its activity through binding to different regulatory subunits. Almost 200 PP1-binding partners have been identified so far, including regulatory subunits, inhibitors, substrate-specifying subunits, and substrates themselves (Bollen et al. 2009; Hendrickx et al. 2009). As a first step to identify PP1 substrates that are critical for spindle checkpoint silencing,

one must identify the regulatory subunit(s) that targets PP1 to kinetochores.

In this issue of *Genes & Development*, Biggins and colleagues (Akiyoshi et al. 2009) have identified such a PP1 regulator in budding yeast. First, Akiyoshi et al. (2009) developed elegant methodology to enrich for and identify kinetochores proteins. Through this analysis, Akiyoshi et al. (2009) identified Fin1p, a protein known previously to localize to and regulate the stability of anaphase spindles, as a novel kinetochore component in metaphase and anaphase. Fin1p binds to PP1 via consensus PP1-binding sites and, in the absence of Fin1p, PP1 levels on kinetochores are significantly decreased, although not abolished. Conversely, PP1 increases the affinity of Fin1p with kinetochores, probably by dephosphorylating Fin1p and thereby disrupting the interaction of Fin1p with 14–3–3 proteins. Overexpression of a *fin1* mutant that cannot be phosphorylated by CDK (*fin-5A*) leads to accumulation of monopolar spindles, yet, surprisingly, this fails to activate the spindle checkpoint. A checkpoint response to these monopolar spindles is restored if the PP1-binding site of *fin1-5A* is mutated, suggesting that overexpression of *fin1-5A* overstimulates the PP1 function in spindle checkpoint silencing. This establishes Fin1p as a key regulator of PP1 in silencing the spindle checkpoint in budding yeast.

Genetic evidence suggests that the Fin1–PP1 complex (^{Fin1}PP1) opposes Aurora kinase activity (Akiyoshi et al. 2009). However, the phosphorylation status of Dam1, a known kinetochore substrate of Aurora, is not affected by ^{Fin1}PP1, suggesting that Fin1p only stimulates the activity of PP1 toward specific kinetochore substrates (Akiyoshi et al. 2009). This might explain why Fin1p is not essential for viability, and raises the possibility that different Aurora substrates are targeted by different pools of PP1, each associated with a specific regulator. That might also explain why lack of Fin1p reduces but does not abolish PP1 localization on kinetochores (Akiyoshi et al. 2009). To date, no functional homologs of Fin1p have been identified in other model systems. Akiyoshi et al. (2009) mention unpublished data suggesting that PP1 interaction with the kinetochore component Spc105/KNL1 is essential for viability. This could mean that Spc105p, at least in budding yeast, is a docking site for the different pools of PP1 phosphatase on kinetochores (Fig. 1). Interestingly, Spc105/KNL1 is also a kinetochore docking site for the vertebrate checkpoint components Bub1 and BubR1 (Kiyomitsu et al. 2007), and is required for proper kinetochore–microtubule attachments in many model systems (Kerres et al. 2007; Wan et al. 2009). Thus, Spc105–PP1 complexes would be at the right place on kinetochores to regulate and coordinate both checkpoint silencing and kinetochore–microtubule stabilization. Importantly, the PP1-binding consensus sites of Spc105p are conserved through evolution.

Fin1p is phosphorylated in vitro by Ipl1/Aurora kinase (Akiyoshi et al. 2009), suggesting that the different pools of PP1 phosphatase on kinetochores might be directly regulated by Aurora itself. This would constitute a positive feedback mechanism by which Aurora limits the activity of its opposing phosphatase on kinetochores.

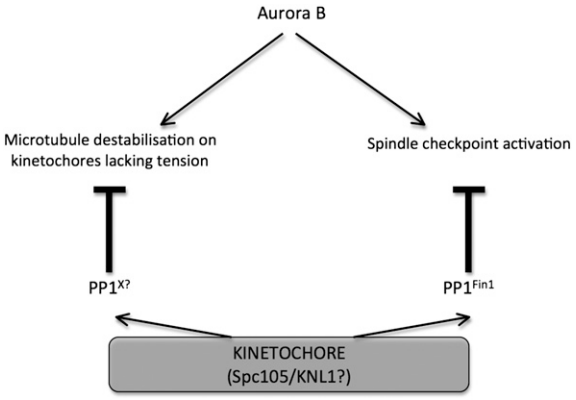

Figure 1. Kinetochore-localized PP1 opposes Aurora B activity to promote anaphase onset. Aurora B regulates the metaphase–anaphase transition in at least two ways: (1) destabilization of kinetochore–microtubule connections failing to produce tension, and (2) activation of the spindle checkpoint. We propose that interaction between a kinetochore component (e.g., Spc105/KNL1) and PP1 is required to counteract both processes. However, distinct substrate-specifying regulators of PP1 oppose different Aurora B functions on kinetochores. Fin1p-associated PP1 ($PP1^{Fin1}$) opposes Aurora B action in the spindle checkpoint and promotes spindle checkpoint silencing. Another as-yet-unknown regulator of PP1 might counteract the Aurora-dependent microtubule-destabilizing activity.

Taken together, these data suggest the following working model. Fin1p and PP1 are interdependent for their kinetochore localization in metaphase, whether or not kinetochores are under tension. On kinetochores lacking tension, Fin1p (and potentially PP1 itself) is inhibited by Aurora kinase, and PP1 cannot silence the spindle checkpoint. On bioriented kinetochores, however, Fin1p becomes active, possibly in a microtubule-dependent manner (Akiyoshi et al. 2009), and potentiates PP1 activity toward as-yet-unknown kinetochore substrates whose dephosphorylation is necessary for spindle checkpoint silencing. Finally, the fact that Fin1p is also required for spindle stability (Woodbury and Morgan 2007) could suggest that spindle checkpoint silencing and spindle stability are somehow coordinated.

Other mechanisms that attenuate checkpoint signals

In vertebrates, other mechanisms have been proposed to disrupt checkpoint-dependent APC/C^{Cdc20} inhibition. $p31^{comet}$ is able to inhibit interactions between Mad2 and Cdc20 and thereby relieve APC/C^{Cdc20} inhibition (Yang et al. 2007). The binding of CENP-E to BubR1 upon microtubule attachment to kinetochores has been proposed to inactivate BubR1 kinase and silence the checkpoint (Mao et al. 2005). Finally, in both vertebrates and yeast, a number of checkpoint components are themselves substrates of the APC/C (Palframan et al. 2006; King et al. 2007b; Qi and Yu 2007; Choi et al. 2009). In particular, recent studies have elegantly shown that human BubR1 is acetylated in metaphase to prevent its degradation by the APC/C (Choi et al. 2009). Preventing

its acetylation destabilizes BubR1 and overrides the checkpoint. Conversely, mimicking BubR1 acetylation prevents mitotic exit. Together, these data suggest that BubR1 degradation by the APC/C is important for checkpoint inactivation (Choi et al. 2009; Yekezare and Pines 2009). However, apart from the CENP-E/BubR1 interaction, it remains unclear how these mechanisms are regulated by chromosome biorientation. We propose that some of these mechanisms merely attenuate checkpoint-dependent APC/C inhibition to allow rapid APC/C^{Cdc20} activation upon chromosome biorientation. In other words, that these mechanisms do not trigger, but rather help, checkpoint inactivation. In doing so, they might contribute to the residual APC/C activity detected upon checkpoint activation (Nilsson et al. 2008; Herzog et al. 2009) and/or spatially restrict checkpoint-dependent APC/C inhibition. This might be particularly important in an open mitosis, where APC/C is found all over the cytoplasm, as opposed to a closed mitosis (such as in yeasts), where the APC/C remains in close proximity to the spindle. Consistent with this, some of these mechanisms are not conserved in yeast (e.g., $p31^{comet}$ and the CENP-E/BubR1 interaction).

A working model for checkpoint silencing

Here we propose a possible scenario for checkpoint activation/silencing. Unattached kinetochores catalyze the initial steps of MCC formation and APC/C^{Cdc20} inhibition. While MCC formation can be relayed in the cytoplasm, several mechanisms act there to attenuate its activity, at least in vertebrates. This could serve two purposes: (1) to spatially restrict MCC formation and activity around the spindle/chromosomes, and (2) to prevent complete APC/C inhibition, which would be harder to relieve. Upon chromosome biorientation, the action of PP1 on kinetochores opposes checkpoint kinases such as Aurora and triggers the local disassembly of anaphase inhibitors (possibly by disrupting Mad2–Cdc20 interactions). Note that it is unclear whether the first pool of APC/C^{Cdc20} to be activated upon chromosome biorientation is formed by association of free Cdc20 with apo-APC/C or by removing checkpoint proteins from APC/C^{MCC}. Nor do we know where these pools reside before and during release from checkpoint arrest. Subsequently, in a positive feedback loop, APC/C^{Cdc20}, assisted by the E2 ubiquitin-conjugating enzyme UBE2S in humans (Garnett et al. 2009), targets checkpoint components such as BubR1 for degradation, and, as cyclin B is degraded, CDK activity drops. This results in full APC/C activity and anaphase onset.

To test this model, two key obstacles need to be overcome: (1) identification of the relevant substrates of PP1 and testing whether its activity contributes to disassembly of Mad2–Cdc20 and/or APC/C^{MCC}, and (2) localization of different pools of APC (±Cdc20) and the design of probes that monitor APC/C^{Cdc20} activity in vivo spatially and over time. APC/C^{Cdc20} activity could be followed indirectly by measuring the drop of CDK activity in vivo; for example, by using FRET probes for CDK activity based on the same principle as those for Aurora B activity (Liu et al. 2009). Identification of Fin1p as a PP1 regulator is a step forward, but many more will be required before the intricacies of checkpoint silencing are fully understood.

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