Focus Review

A quantitative systems view of the spindle assembly checkpoint

Andrea Ciliberto[1],* and Jagesh V Shah[2],*

[1]IFOM—Firc Institute of Molecular Oncology, Milan, Italy and
[2]Department of Systems Biology, Harvard Medical School and
Renal Division, Brigham and Women's Hospital, Boston, MA, USA

The spindle assembly checkpoint acts to delay chromosome segregation until all duplicated sister chromatids are captured by the mitotic spindle. This pathway ensures that each daughter cell receives a complete copy of the genome. The high fidelity and robustness of this process have made it a subject of intense study in both the experimental and computational realms. A significant number of checkpoint proteins have been identified but how they orchestrate the communication between local spindle attachment and global cytoplasmic signalling to delay segregation is not yet understood. Here, we propose a systems view of the spindle assembly checkpoint to focus attention on the key regulators of the dynamics of this pathway. These regulators in turn have been the subject of detailed cellular measurements and computational modelling to connect molecular function to the dynamics of spindle assembly checkpoint signalling. A review of these efforts reveals the insights provided by such approaches and underscores the need for further interdisciplinary studies to reveal in full the quantitative underpinnings of this cellular control pathway.

Introduction

The goal of mitosis is to take the duplicated genome, in the form of chromosomes, and ensure its equal distribution to each daughter cell. This distribution is carried out by the mitotic spindle, a complex machine that captures the duplicated chromosomes at their centromeres and segregates them. The fidelity and control of this process is governed

*Corresponding authors. A Ciliberto, IFOM—Firc Institute of Molecular Oncology, Via Adamello 16, 20139, Milan, Italy.
Tel.: +390 257 430 3253; Fax: +390 257 430 3231;
E-mail: andrea.ciliberto@ifom-ieo-campus.it or JV Shah, Department of Systems Biology, Harvard Medical School and Renal Division, Brigham and Women's Hospital, 4 Blackfan Circle, Boston, MA 02115, USA.
Tel.: +1 617 525 5912; Fax: +1 617 525 5965;
E-mail: jagesh_shah@hms.harvard.edu

by the spindle assembly checkpoint, a cellular pathway that delays chromosome segregation, or anaphase, until they have all been appropriately captured by the mitotic spindle. Failure of the spindle assembly checkpoint results in gain and loss of chromosomes, or aneuploidy, a condition associated with malignancy and birth defects. Given its role, it is not surprising, but yet striking, that the spindle assembly checkpoint can delay anaphase in response to a single uncaptured chromosome, exhibiting excellent sensitivity. Once this last chromosome attaches, the spindle assembly checkpoint disengages and rapidly promotes anaphase onset. High fidelity and speed are usually competing design constraints in man-made machines, and as such the underlying logic and quantitative mechanisms of the spindle assembly checkpoint are of interest to life scientists and physical scientists alike. Here, we present a systems view of the spindle assembly checkpoint in which we modularize the complexity of the components into the key communicating elements and consider the measurements and modelling of these elements that have started to reveal the quantitative basis of this exquisite cellular control mechanism.

Spindle assembly checkpoint signalling—a primer

The basic schema of the spindle assembly checkpoint is a balance between an inhibitory signal to prevent anaphase and the activity of the anaphase-promoting machinery (Figure 1). The key site in the production of the inhibitory signal is the kinetochore, a protein complex that assembles at the centromere of mitotic chromosomes (reviewed in an accompanying contribution from Santaguida and Musacchio). The unattached kinetochore acts as a catalytic scaffold for inhibitor production. As cells enter mitosis, all kinetochores are unattached and generate a signal that acts to prevent the onset of anaphase through direct inhibition of the anaphase promoting machinery (Figure 1A). The capture of chromosomes at both sister kinetochores, by microtubules of the mitotic spindle, silences the production of this signal (Figure 1B and C). The stoppage in inhibitor production leads to the activation of anaphase-promoting activity. The origin of the anaphase-promoting activity is an E3 ubiquitin ligase, aptly named the anaphase-promoting complex or APC/C (King *et al*, 1995; Sudakin *et al*, 1995). To promote anaphase onset the APC/C, activated by its cofactor Cdc20, ubiquitinates (Fang *et al*, 1998a), and thereby targets for destruction by the proteasome, cyclin B and securin (Glotzer *et al*, 1991). Loss of cyclin B begins the program of mitotic exit through the reduction of cyclin-dependent kinase (Cdk1) activity. Loss of securin releases the activity of a protease known as separase that cleaves the 'molecular glue', or cohesin complexes, which bind replicated chromatids together
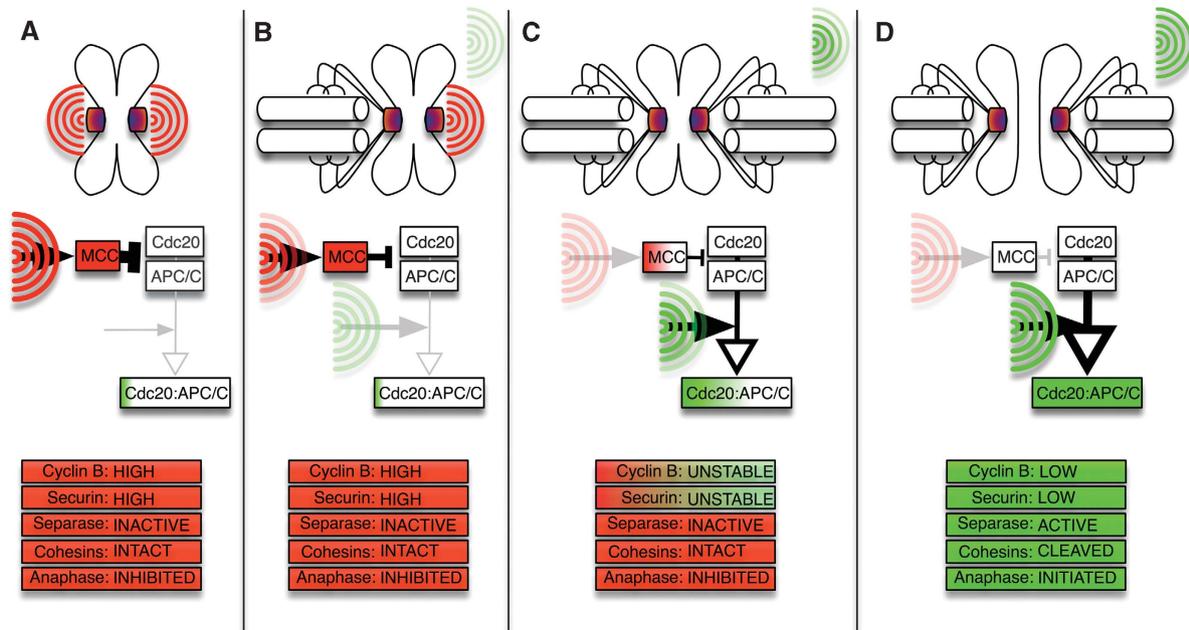
Figure 1 Schematic view of spindle assembly checkpoint signalling. (**A**) Cells enter mitosis with unattached kinetochores that actively generate inhibitory signals (strong red alarm signal) to prevent APC/C activation. This stabilizes the high levels of cyclin B and securin that prevent anaphase onset. (**B**) Attachment of spindle microtubules to unattached kinetochores locally turns off kinetochore-mediated inhibition, but cytoplasmic inhibition, potentially diminished, is still supported by other unattached kinetochores (weaker red signal). The progressive attachment of microtubules generates a weak signal in the cytoplasm that promotes the disengagement of the checkpoint (weak green alarm signal) (**C**) Capture of all chromosomes results in the complete loss of signal generation from kinetochores (weakest red signal), permitting the greater relief of inhibition on the APC/C in the cytoplasm (stronger green alarm). Activation of the APC/C promotes the destabilization of cyclin B and securin. (**D**) Sufficient loss of substrates (cyclin B and securin) promotes the activation of separase and cleavage of the cohesins permitting the onset of anaphase and segregation of the sister chromatids.

(Figure 1D). This transition to anaphase promotes both the segregation of the genetic material, and exit into the subsequent cell cycle for both progeny cells. The spindle assembly checkpoint delays APC/C activation until all kinetochores are properly attached to microtubules.

The generation of the inhibitory signal and its mode of inhibition have been widely studied (reviewed in Musacchio and Salmon, 2007). Less well understood are the mechanisms for relieving the inhibition of the APC/C and permitting the transition to anaphase. Together, these activities, inhibition on the one hand and release of that inhibition on the other, must support the widespread observation of a single unattached kinetochore delaying the onset of anaphase. Moreover, the coupling of these activities and their relative dominance must be controlled entirely through kinetochore attachment to permit the rapid transition to anaphase on kinetochore attachment. Each of these activities: inhibitor generation, release from inhibition and kinetochore attachment are themselves complex signalling pathways involving a myriad of molecular components. A systems view of spindle assembly checkpoint signalling focuses our attention onto the communication between signalling modules that are likely to govern the quantitative dynamics of this pathway.
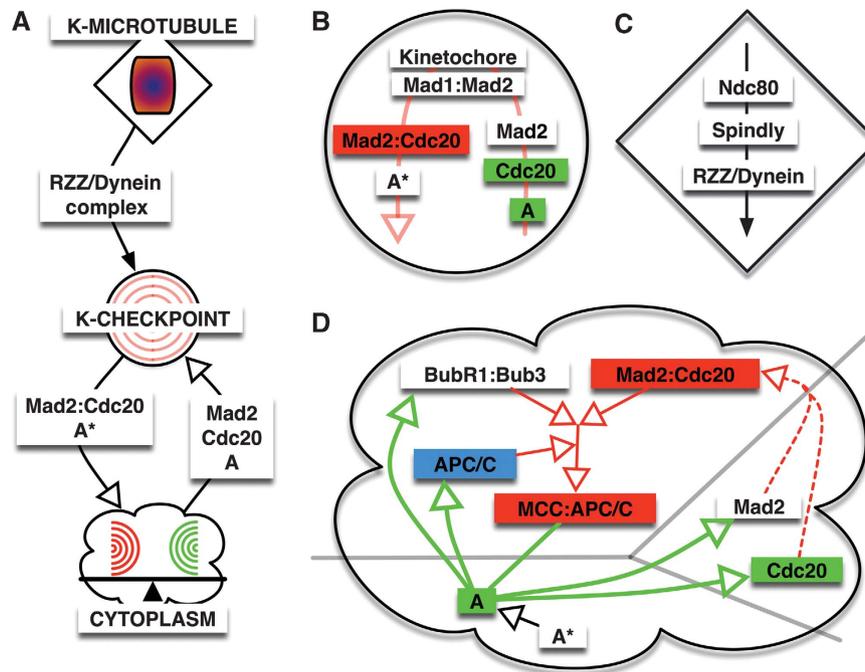
A modular view of spindle assembly checkpoint signalling

The spindle assembly checkpoint requires the coordination between many signalling pathways. Unattached kinetochores produce a signal that informs the cytoplasm of the lack of chromosome attachment. Once engaged, the attachment machinery promotes the silencing of the kinetochore-based signalling platform. Finally, the fulfillment of a state of kinetochore attachment for all chromosomes must be transmitted, either actively or passively to the cytoplasm to activate the anaphase-promoting machinery. From this simple description we can identify three major modules: the kinetochore-localized signalling platform, the spindle attachment machinery and the cytoplasmic activities associated with APC/C activity (Figure 2A). The modules act to abstract internal molecular interactions, some of which are still unknown, in favour of those inter-module interactions that regulate rapid information transfer and are likely to support the observed dynamics.

Kinetochore-mediated signal generation

The assembly of the kinetochore is a complex process that involves a host of proteins (see this issue Santaguida and Musacchio and Musacchio and Salmon, 2007). The checkpoint elements of the kinetochore were originally revealed in a set of seminal budding yeast screens that gave rise to the mitotic arrest deficient (Mad 1, 2 and 3) and budding inhibited by benzimidazole (Bub1 and 3) genes that sparked the molecular understanding of the checkpoint (Hoyt *et al*, 1991; Li and Murray, 1991). Central to these gene products is their specific localization or enrichment at unattached kinetochores, as first revealed by Chen and Murray and Li and Benezra for the vertebrate orthologue of Mad2 (Chen *et al*, 1996; Li and Benezra, 1996). The inhibitor generation signalling paradigm of the kinetochore was first demonstrated by

Figure 2 Modular organization of the spindle assembly checkpoint. (**A**) The interactions between the modules that comprise the spindle assembly checkpoint. The K-microtubule module represents the proteins at the kinetochore that control microtubule attachment. The K-checkpoint module, the network of proteins at the kinetochores that generate the inhibitory flux of Mad2:Cdc20 and A*. Finally, the cytoplasm module represents the reactions of MCC:APC/C association and release taking place in the cytoplasm—the balance between APC/C inhibition and its release. The filled arrows represent the molecular interactions controlling the activity of the scaffold at the unattached kinetochore. The open arrows indicate net fluxes, which result in the generation of Mad2:Cdc20 complexes from the unattached kinetochores and the release of free Cdc20 through active dissociation within the cytoplasm. (**B**) The spindle assembly checkpoint signalling elements of the kinetochore (K-Checkpoint) catalyse, through the Mad1:Mad2 scaffold, the formation of Mad2:Cdc20 complexes. In this representation, the kinetochore can also modulate the level of cytoplasmic MCC:APC/C dissociation activity through the proposed A to A* modification. Red complexes act to inhibit APC/C activity, whereas green activate. (**C**) The microtubule attachment module of the kinetochore (K-Microtubule) involves the microtubule attachment complex, Ndc80, and the Dynein-binding protein Spindly. The action of Spindly and the Rod–Zw10–Zwilch complex (RZZ) controls indirectly, through microtubule attachment, kinetochore-mediated inhibitor generation. (**D**) The cytoplasm has three submodules. The first forms the MCC:APC/C inhibitory complex from the Mad2:Cdc20 complex and other cytoplasmic components (BubR1:Bub3 and APC/C). The second actively dissociates the inhibited APC/C into the component parts, through the activity of A. Note that A* (inactive) is converted to A (active) in the cytoplasmic module. The third component comprises non-kinetochore mechanisms for generation of the Mad2:Cdc20 complex, specifically through cytoplasmic amplification from Closed Mad2 complexes in the cytoplasm (Mad2:Cdc20, MCC (not shown) and MCC:APC/C). This last reaction is represented with a dashed line.

Rieder and colleagues who through the laser-mediated ablation of the last unattached kinetochore and the resulting precocious onset of anaphase identified the kinetochore as the source of the anaphase inhibitory signal (Rieder *et al*, 1995). Finally, the observation of Mad2 turnover at unattached kinetochores (Howell *et al*, 2000) solidified the widely held model of checkpoint signalling by which the unattached state of the kinetochore is transmitted to the cytoplasm through the transient recruitment and activation of Mad2 (Figure 2B). By the time of the demonstration of kinetochore turnover, Mad2 had already been shown to interact with Cdc20, the activator of the mitotic APC/C, and to inhibit APC/C activity (He *et al*, 1997; Li *et al*, 1997; Hwang *et al*, 1998; Kallio *et al*, 1998; Kim *et al*, 1998; Wassmann and Benezra, 1998; Fang *et al*, 1998b). Moreover, in seminal work by Sudakin *et al* (2001), a potent inhibitory complex, the mitotic checkpoint complex (MCC), was found to contain Mad2, Cdc20, BubR1/Mad3 and Bub3 proteins, all found enriched at unattached kinetochores. Further studies revealed that all components of the MCC turnover at unattached kinetochores (Howell *et al*, 2000, 2004; Kallio *et al*, 2002; Shah *et al*, 2004) further supporting the role of the unattached kinetochore as the catalytic platform for inhibitor production.

Detailed structural studies demonstrated that the first step in the formation of this inhibitor occurs through the conformational activation of Mad2 (Xia *et al*, 2004; De Antoni *et al*, 2005; Yang *et al*, 2008). Structural studies of the Mad2 conformational change, pioneered by the laboratories of Yu and Musacchio, showed that the Mad1-bound form of Mad2 (Closed or N2), can induce a second Mad2 molecule, normally in the Open or N1 conformation in the cytoplasm, to acquire the active conformation (Closed or N2). Thus activation requires a transient dimerization (Mapelli *et al*, 2007; Yang *et al*, 2008) that occurs at the unattached kinetochore, in which Mad2 is in the closed form bound to Mad1 (Mapelli *et al*, 2006). This transient dimerization was observed in living cells by Shah and colleagues who demonstrated that only a proportion (∼50%) turned over at kinetochores and that the remainder was stable, presumably bound to stable Mad1 (Shah *et al*, 2004). Activation permits Mad2 to bind Cdc20 resulting in a Mad2:Cdc20 complex incapable of activating the APC/C. The complete MCC also includes the checkpoint proteins BubR1 (Mad3 in lower organisms) and Bub3 that bind the Mad2:Cdc20 complex at the kinetochore or in the cytoplasm and it is this complex that acts to inhibit APC/C activity (Millband and Hardwick, 2002; Davenport *et al*, 2006; Essex *et al*, 2009; Kulukian *et al*, 2009).

It is important to note that a number of other proteins (Mps1, Bub1, Aurora B, Plk1, CENP-E, CENP-F, etc.), and in particular kinases, have been shown to have a function in the checkpoint. In some cases, these proteins may be required for assembly of the catalytic platform itself. However, it is also possible that these proteins have a more direct function in APC/C inhibition, or its relief. For example, the checkpoint kinase Bub1, has a key function in recruitment of checkpoint proteins to kinetochores (Meraldi and Sorger, 2005) but also can phosphorylate Cdc20 to prevent it from interacting with APC/C or spindle assembly checkpoint components potentially acting to buffer Cdc20 levels during spindle assembly checkpoint activation (Tang et al, 2004). Such distinct activities in spindle checkpoint signalling can also be proposed for Mps1, Aurora B and Plk1 kinases. As such, in our representation of the modules comprising the spindle assembly checkpoint, protein activities (like those described for Bub1) can be split between the assembly of the catalytic scaffold (Figure 2B) and 'A', an abstract quantity whose activity directly regulates APC/C inhibition (Figure 2B and D) through an alternative pathway, depicted here as a regulator of MCC:APC/C dissociation.

At its core, this module takes as input Cdc20 and Mad2 and a hypothetical activity 'A', that acts to release APC/C inhibition, and produces an inhibitory Mad2:Cdc20 complex and 'A*', an inactive form of A. Both outputs act to inhibit APC/C activity and thus prevent anaphase onset. The quantitative production rates of these species are the central quantities of interest that emerge from this module and must ultimately account for single kinetochore inhibition.

Microtubule-binding interface and kinetochore-localized signal silencing machinery

In addition to the generation of the checkpoint signal, the kinetochore also acts to capture and stabilize spindle microtubules, ultimately using them to power transport of sister chromatids to the presumptive daughter cells. The molecular components involved in this process are numerous, but restricting our focus to the spindle checkpoint permits the definition of an interface between the microtubule-binding components and spindle checkpoint components of the kinetochore. Importantly, these components at the interface are candidates to regulate the activity of the catalytic scaffold permitting the silencing of the signal generation on microtubule attachment. Key candidates for this interface are the Ndc80 and the Rod–Zw10–Zwilch (RZZ) complexes (Figure 2C).

The Ndc80 complex is a major microtubule-binding component of the kinetochore and is widely conserved in evolution. Reduction of Ndc80 complex levels (through the modulation of the Hec1 subunit) results in the dramatic loss of stable spindle attachments (Cheeseman et al, 2006; DeLuca et al, 2006) but also diminishes Mad2 (Martin-Lluesma et al, 2002; Guimaraes et al, 2008) and RZZ complex recruitment to kinetochores (Lin et al, 2006; Miller et al, 2008). Surprisingly, the checkpoint remains active under this reduction of recruited Mad2, and Mad2 is recruited to normal levels if cells are subjected to spindle poisons (Guimaraes et al, 2008). As expected, complete loss of the Ndc80 complex results in the complete absence of a mitotic checkpoint underscoring the minimal requirement for Mad2 recruitment to establish and maintain a checkpoint arrest (Meraldi et al, 2004).

In addition to Ndc80, Mad2 localization and kinetochore-mediated checkpoint activation is dependent on the RZZ complex (Kops et al, 2005; Griffis et al, 2007). This complex, which is present only in metazoans, is recruited to kinetochores to establish a docking site for the molecular motor Dynein (Starr et al, 1998) mediated through the recently identified protein Spindly/SPDL-1 (Griffis et al, 2007; Gassmann et al, 2008; Yamamoto et al, 2008). Once Dynein is engaged at the kinetochore, it interacts with spindle microtubules. Notably, when the microtubule interaction is stabilized, the kinetochore, or more precisely the Mad2 recruitment portion of the catalytic scaffold, is carried away by the Dynein–RZZ complex along the captured spindle microtubules to the spindle poles. This mechanism is critical as it provides a local mechanism for signal silencing that otherwise maintains active checkpoint signalling in the presence of attached kinetochores (Howell et al, 2001; Buffin et al, 2005; Sivaram et al, 2009). Given this role in streaming Mad2, and a portion of the catalytic scaffold, away from attached kinetochores, it is not surprising that the RZZ complex is also required for the localization of Mad2 and an intact checkpoint.

Together, RZZ and the Ndc80 complexes regulate both microtubule attachments and the recruitment of Mad2. Recent work from Gassmann and colleagues has provided a critical link between RZZ and Ndc80 that depends on the Spindly protein (Gassmann et al, 2008). Through mediation of the maturation of the microtubule attachment, Spindly is proposed to determine the handoff of the microtubule from RZZ–Dynein to the Ndc80 complex and is thus poised to simultaneously regulate microtubule attachments and the inhibitor generation activity at the kinetochore (Civril and Musacchio, 2008) (Figure 2C). This emerging picture provides a key connection between microtubule attachment and the local inactivation of inhibitor generation at the kinetochore.

Cytoplasmic activities of APC/C activity and regulation

Although the kinetochore has been of tremendous interest in checkpoint dynamics, a significant portion of checkpoint activity also takes place through cytoplasmic interactions that remain poorly understood. The cytoplasmic 'module', as such, has many potential interactions with the kinetochore reflecting a complex communication with the unattached kinetochore that are likely to go beyond the reliance on a single diffusible stoichiometric inhibitor.

As described above, the kinetochore can provide a scaffold for the generation of the Mad2:Cdc20 complex that can become a full MCC complex either at the kinetochore (Howell et al, 2004; Shah et al, 2004) or in the cytoplasm (Essex et al, 2009; Kulukian et al, 2009) by binding the BubR1:Bub3 complex (Figure 2D). As the APC/C is not specifically localized within cells, although it is enriched on the spindle, at spindle poles (Huang and Raff, 2002) and centromeres (Acquaviva et al, 2004), it is widely held that the diffusion of this complex from the kinetochore into the cytoplasm is critical for forming the inhibitory MCC:APC/C complex (Sudakin et al, 2001; Herzog et al, 2009). Once bound to the APC/C, the MCC acts as a pseudosubstrate inhibitor with BubR1/Mad3 having a key function in inhibiting the recruitment of anaphase targets to the APC/C that would otherwise be recruited by Cdc20 (Burton and Solomon, 2007).

Once formed, the spontaneous dissociation rate of the MCC:APC/C complex is small as observed *in vitro* and in mitotic extracts, indicating a tight interaction (Reddy *et al*, 2007). However, the presumed rate of dissociation, indirectly observed *in vivo* after all kinetochores having attached, is relatively rapid (Clute and Pines, 1999; Morrow *et al*, 2005; Braunstein *et al*, 2007; JVS unpublished data). The dissociation of the MCC from the APC/C, and the deactivation of Mad2, has been proposed by Reddy and colleagues to occur through Cdc20 ubiquitination in the context of the MCC:APC/C in complex with its E2 enzyme UbcH10 (Reddy *et al*, 2007). This process may itself be balanced by deubiquitination by the deubiquitinating enzyme USP44 (Stegmeier *et al*, 2007). The Cdc20 modification is a non-degradative ubiquitination, which is proposed to break the complex formed between Mad2 and Cdc20, a role played by the generic molecule 'A' (Figure 2D). Given that the binding of Cdc20 and Mad2 is expected to be a spontaneous process in living cells, this piece of data provides a potential source of energy needed to destabilize the complex (Simonetta *et al*, 2009).

It is tempting to integrate these observations into a model of the checkpoint whereby unattached kinetochores not only control the formation of the inhibitor but also its dissociation, as is proposed by the modulation of A by the kinetochore catalytic scaffold. It can be argued that with this wiring, the spindle assembly checkpoint would guarantee a more effective inhibition and faster release of Cdc20 as compared with a system in which signalling only controls the formation of the inhibitor (see Box 1 for a more detailed description).

The proposed dissociation pathway has been brought into question by recent data suggesting that Cdc20 ubiquitination is not required for checkpoint exit but instead to keep the level of Cdc20 low during spindle assembly checkpoint activation (Nilsson *et al*, 2008) as has been observed in other organisms (Pan and Chen, 2004). Although the details of this mechanism remain to be clarified, the dissociation rate of the MCC:APC/C complex more than the mechanism *per se*, modulates the balance of inhibition and release and determines the basis for single kinetochore sensitivity and the timing of spindle assembly checkpoint inactivation.

Inhibitor generation has also been implicated within the cytoplasm in which the Mad2:Cdc20 complex generated at the unattached kinetochore, which also contains a Closed (or N2) Mad2 molecule, can induce Mad2 activation by dimerization. Through this reaction, it can hypothetically act to generate new active Mad2 in the cytoplasm through an autocatalytic loop (De Antoni *et al*, 2005). Such activity has been observed *in vitro*, but not yet *in vivo* (Simonetta *et al*, 2009). Such a cytoplasmic amplification could act as a non-kinetochore source of Mad2:Cdc20 complexes to aid in inhibition of the APC/C (Figure 2D).

The combination of the dissociation of the inhibitory complex and the non-kinetochore-mediated generation of APC/C inhibitors underscores the complex role of the cytoplasmic module in checkpoint activation and silencing.

Together, these modules identify the critical interfaces by which the kinetochore, microtubules and the cytoplasm exchange information to determine spindle assembly checkpoint activity. As described below, quantitative measurements and computational modelling efforts have focused on these interfaces to provide insight into the dynamics that regulate this pathway.

Box 1 Dynamical regulation of inhibitor generation and dissolution: Faucets, Sinks and Plugs
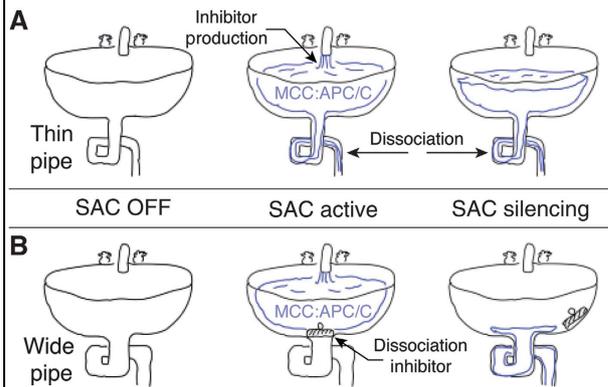
Box 1 It is well known and accepted that the spindle assembly checkpoint helps the formation of Mad2:Cdc20, and subsequent MCC:APC/C complexes through the activity of Mad1:Mad2. Recent evidence suggests that the checkpoint could also act through stabilizing the MCC:APC/C complex. Reddy, Stegmeier, Rape and collaborators showed that the MCC:APC/C complex can be dissociated by ubiquitination (Reddy *et al*, 2007), a reaction opposed by the deubiquitinase USP44 (Stegmeier *et al*, 2007), whose activity has been found high in mitotic extract. It is not known whether the checkpoint indeed activates USP44 (a potential mechanism for A to A* conversion in Figure 1). It is, however, interesting to investigate the dynamical consequences of a system in which the checkpoint only induces the formation of MCC:APC/C as compared with a system in which it both induces its formation and stabilizes it. The two can be described metaphorically by a sink, in which MCC:APC/C is represented by the water accumulated in the basin. If the spindle checkpoint acts simply by favouring the production of MCC:APC/C—panel **A**, opening of the faucet—we have to assume that the spontaneous dissociation of MCC:APC/C must be small compared with the influx of MCC:APC/C for the checkpoint to efficiently inhibit APC/C (thin pipe). As a consequence, the silencing of the checkpoint will necessarily be dictated by the slow rate of disappearance of MCC:APC/C resulting in a long delay between the switching off the kinetochore (faucet is closed) and spindle assembly checkpoint silencing (basin empty). If, on the other hand, the spindle assembly checkpoint not only contributes with 'faucet' molecules (MCC:APC/C), but also with 'plug' molecules that stabilize MCC:APC/C—panel **B**—the dynamics can be quite different. Here, we can imagine that a fast rate of MCC:APC/C dissociation (wide pipe) is masked by the activity of the checkpoint (plug in wide pipe). As soon as the kinetochores are attached, not only does the influx of MCC:APC/C cease (faucet is closed) but the inhibition is relieved as well (plug is removed) and Cdc20 can be re-activated (basin empty) with a much faster pace. Here, we discuss this activity through the species 'A' that has yet to be verified or provided with a molecular correlate. However, the emerging modelling and molecular data suggest that such a pathway is likely to be present.

Quantitative observations of spindle assembly checkpoint activity

The scarcity of quantitative data often hinders the understanding of cellular systems from a systems perspective. The spindle assembly checkpoint, however, is a notable exception. This field has amassed a substantial amount of quantitative data, on which mathematical models have developed. In this section, we will review some of the most significant quantitative data available for the spindle assembly checkpoint, whereas in the next section, we will

describe how these data have been used by modellers to provide a systems perspective of the spindle assembly checkpoint.

APC/C reactivation kinetics

The timing of mitosis and in particular anaphase onset has been the subject of study for over a century (reviewed in Mazia, 1961). The delay of anaphase with respect to the attachment of the last kinetochore was measured in detail by seminal experiments of Rieder and colleagues. Rieder placed the timing of last kinetochore attachment to anaphase onset at ∼25 min by observation of rat kangaroo (Potorous tridactylus) kidney epithelia cells (Rieder et al, 1995). This interval spans a number of key biochemical steps: (1) the release of APC/C inhibition, (2) ubiquitination and degradation of cyclin B and securin, (3) activation of separase, (4) degradation of cohesin and (5) initiation of the anaphase movements. As such, we can place the reactivation time of the APC/C at a maximum of ∼25 min (for reactivation in 10 min $k_{diss} \sim 0.0017/s$) if this were in fact the rate-limiting step. For a mammalian cell in mitosis (∼100 nM APC/C, all inhibited, 6 pL volume) this would imply that ∼600 molecules of MCC:APC/C dissociate per unit time (Sear and Howard, 2006). In fact, Howell and colleagues were able to further refine the time between the attachment of the last kinetochore to anaphase onset to ∼12 min based on the last detectable kinetochore-bound Mad2 until the onset of anaphase (Howell et al, 2000), suggesting that APC/C dissociation actually occurs even faster.

Inhibitor production

In the presence of one or more unattached kinetochores, the APC/C dissociation rate must be balanced by inhibitor production. In the simplest scheme whereby Cdc20 binds to Mad2 turning over at the unattached kinetochores, the quantification of the generation rate of Mad2:Cdc20 requires two measurements: (1) the number of molecules of Mad2 and Cdc20 at unattached kinetochores and (2) the turnover rate of these molecules at unattached kinetochores. These measurements have been made by a number of groups (Howell et al, 2000, 2004; Kallio et al, 2002; Shah et al, 2004). Together, they observe a surprisingly small number of Mad2 molecules at an unattached kinetochore (∼1300) (Howell et al, 2000) whose turnover occurs with fairly rapid dynamics ($t1/2 \sim 10$–25 s). A simple calculation (assuming 100% efficiency of complex formation) estimates the production rate of 30–60 Mad2:Cdc20 complexes/sec/kinetochore, which for a stoichiometric inhibitor would be unable to match the estimated APC/C dissociation (JVS, unpublished data). This deficit, first described by Sear and Howard (2006), implies the presence of other cellular mechanisms in checkpoint signalling. The computational models described below evaluate potential mechanisms to reduce this deficit and maintain single kinetochore sensitivity.

## Conceptual and quantitative insight provided by computational modelling

Mathematical models of the cell cycle have mainly focused on the description of the combination of positive and negative feedback loops that give rise to the cell cycle engine that generates peaks of the cyclin proteins that drive cells in and out of mitosis (Tyson et al, 2002; Ingolia and Murray, 2004). Although checkpoints are represented within these models, the fine mechanisms whereby a structural event, such as DNA damage or the presence of unattached microtubules, triggers a chain of reactions that impinges on cell cycle progression requires alternative modelling strategies. A closer representation of biophysical constraints such as forces or spatial localization is required in this case. Some of these models, with particular emphasis on microtubule dynamics, have recently been reviewed by Mogilner et al (2006). Here, we account for mathematical analyses of the spindle assembly checkpoint that have been proposed in recent years, ranging from models structured on generic molecular networks (Doncic et al, 2005; Sear and Howard, 2006; Mistry et al, 2008), to models aimed at reproducing the spindle assembly checkpoint network in molecular detail. Molecular models either include the full network (Ibrahim et al, 2008a, b, 2009) or some smaller elements (Simonetta et al, 2009). Many of these efforts are structured around the modular framework presented above and use the many quantitative measurements described earlier. Here, we consider these contributions and the insight that such approaches can provide to our understanding of checkpoint dynamics.

Biophysical models

The pioneering work of Doncic et al (2005) addressed possible molecular mechanisms for the spindle assembly checkpoint network using biophysical processes and measurements without the explicit identification of molecular components. This approach led to the production of what we call biophysical models.

Doncic and colleagues argued, as above, that any model of the spindle assembly checkpoint had to recapitulate two properties: the capability of the spindle assembly checkpoint to robustly halt cell cycle progression, and its quick disengagement once all kinetochores are attached. Using observations from the closed mitosis of budding yeast, these requirements meant that successful molecular mechanisms were asked to have at least 95% of the cellular Cdc20 sequestered (1000 molecules in a spherical nuclear volume 1 μm in radius, or ∼130 nM). The calculations were done assuming one unattached kinetochore (10 nm in radius) placed at the centre of a simple spherical geometry and simple diffusion (diffusion coefficient ∼1 μm²/s). Moreover, they required that >90% of Cdc20 (or equivalently the APC/C) would be re-activated 3 mins after the last kinetochore was attached. First, they tested the simplest possible model for the spindle assembly checkpoint, called 'direct inhibition' (Figure 3A) whereby Cdc20 molecules are inhibited by recruitment to the unattached kinetochore (Acquaviva et al, 2004) and activated constitutively in the cytoplasm. Making the assumption that all Cdc20 molecules passing by the kinetochore are inhibited, they show that direct inhibition cannot maintain an anaphase delay because of the disparity between Cdc20 visitation rate and cytoplasmic reactivation rate—molecules get reactivated quicker than they can visit the kinetochore. A second possibility tested by Doncic et al is 'cytoplasmic amplification', a model in which inhibited molecules of Cdc20 in the cytoplasm induce the further inhibition of other Cdc20 molecules. Such a possibility, reminiscent of models proposed by De Antoni et al (2005) (but see later
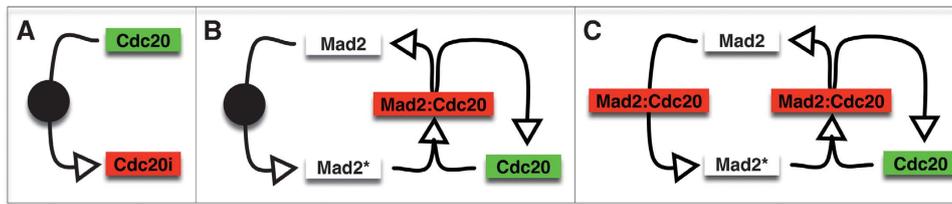
Figure 3 Molecular interpretation of wiring diagrams proposed in biophysical models. (**A**) In the simplest model, 'direct inhibition model' in Doncic *et al* (2005), the checkpoint (here Cdc20—active) is inhibited (Cdc20i—inactive) by direct contact with the kinetochore (black dot in the figure). In red, inhibited species, in green, the active ones. (**B**) According to the 'indirect inhibition model' (Doncic *et al*, 2005), kinetochores produces an active species (Mad2*) that inhibits Cdc20 through a complex that is dissociated in the cytoplasm. (**C**) The 'Mad2 template model' postulates that the role of the kinetochore in (B) can be played by the complex Mad2:Cdc20 in the cytoplasm. In this description, we include the activated species Mad2*, which is missing in the original formulation of the template model (De Antoni *et al*, 2005). The combination of (B, C) gives rise to the full template model. Of note is the autocatalytic loop that is present in (C).

for a more thorough comparison), displays tight inhibition. However, in this formulation of the autocatalysis, the checkpoint cannot be turned off as even after the kinetochore is silenced the cytoplasmic inhibitory activity remains potent. Finally, they explore a model by which a stoichiometric inhibitor can be generated at the kinetochore (Figure 3B). The inhibitor binds to and inhibits Cdc20 and the resulting complex undergoes dissociation at some fixed rate. In this case, the kinetochore can 'overproduce' inhibitor to buffer any free Cdc20 that may form in the cytoplasm. Once the kinetochore is silenced by microtubule attachment, the dissociation activity rapidly reactivates Cdc20 to permit checkpoint exit. This 'indirect inhibition' model matches all the requirements laid out by Doncic and colleagues for an efficient spindle assembly checkpoint. Of note is that this scheme is similar, in principle, to the production of MCC, a stoichiometric inhibitor, and its binding to and inhibition of the APC/C.

Using these simulations, Doncic and colleagues lay out a simple scheme to simulate checkpoint signalling and provide the cornerstone in quantitative modelling of the spindle assembly checkpoint. Subsequent analyses, described below, follow closely from this approach. A drawback with respect to the specific conclusions of Doncic and colleagues is the choice of parameters, particularly those that may not reflect the *in vivo* dynamics. For example, the exact number of Cdc20 molecules that need to be sequestered during spindle assembly checkpoint activation has not been measured. Fewer Cdc20 molecules could provide an opportunity for one of the earlier models to emerge as appropriate, whereas more Cdc20 molecules may cause even the indirect inhibition to fail. This point gains importance given that Cdc20 is destabilized when the spindle assembly checkpoint is engaged (Pan and Chen, 2004; Nilsson *et al*, 2008), and also inhibited by phosphorylation (Tang *et al*, 2004) potentially reducing the requirement of total Cdc20 sequestration or Cdc20:APC/C inhibition. Furthermore, they did not take into account a finite number of binding sites for protein interaction at the kinetochore. Using an 'infinite flux' assumption, that is all molecules that visit the kinetochore can be bound and modified, imparts the kinetochore with a greatly overestimated catalytic power, particularly given that measurements in living mammalian cells have demonstrated that the kinetochore production rate of a Mad2 inhibitor is in fact quite low.

Using the Doncic work as a starting point, Sear and Howard introduced measurements that had been made in

mammalian cells and mammalian cell dimensions to their analysis (Sear and Howard, 2006). Using data from photobleaching experiments and kinetochore protein abundance (Howell *et al*, 2000, 2004; Kallio *et al*, 2002; Shah *et al*, 2004) and estimates of APC/C reactivation they confirmed that direct inhibition (Figure 3A) is not feasible. Moreover, they demonstrated, as described above, that the simple balance of inhibitor production from a single kinetochore, again based on cellular measurements, and complex dissociation would not support anaphase delay even in an indirect inhibition model (Figure 3B). To address the discrepancy in supporting a checkpoint signal, Sear and Howard (2006) suggested additionally that the cytoplasm may also contribute to the generation of the wait anaphase signal, although not through autocatalysis. Here, they propose the production of an inhibited species from unattached kinetochores that can catalyse the production of a qualitatively different inhibitor in the cytoplasm, but that this latter inhibitor itself cannot catalyse further inhibitors. That is, the kinetochore-produced component X can generate inhibitor Y in the cytoplasm, but that Y cannot generate any further inhibitory molecules, so-called one-step amplification. In this way, they avoid the problem of exiting the checkpoint associated with the autocatalytic cytoplasmic amplification model, since the kinetochore has more direct control over the amplification. The model proposed gives good results in terms of strength of inhibition and speed of release, but unfortunately cannot be reconciled at this time with the molecular players that are known to have a function in the spindle assembly checkpoint.

More recently, Mistry and collaborators elaborated a modification of the model proposed by Sear and Howard (Mistry *et al*, 2008) that presents the first attempt to describe the dynamics of microtubule attachment to the kinetochores, an important step in making spindle assembly checkpoint models closer to biological reality.

In summary, biophysical models have proven useful in developing a framework for the systems behaviour of the spindle assembly checkpoint. They have developed strong evidence that the spindle assembly checkpoint is unlikely to work through a mechanism of direct inhibition and identified subtleties related with the presence of a cytoplasmic catalytic activity that supports the checkpoint. The demonstration of the failure of the indirect inhibition model in mammalian cells implies that while our intuition regarding the mechanism may be sound in principle, substituting in actual measurements reveals a significant gap in our quantitative understanding of the checkpoint. As such, these biophysical

models may provide an important function in testing hypotheses for quantitative plausibility rather than revealing specific molecular pathways.

Molecular models

Given their poor characterization in molecular terms, biophysical models are very useful to understand the systems level behaviour but often cannot provide a clear connection to a molecular mechanism. Unlike biophysical models, 'molecular' models rely on known molecular interactions and rate constants to simulate spindle checkpoint signalling. As such, these models require extensive knowledge of reaction rates, concentrations and network topologies: pre-conditions that are not always fulfilled in the case of the spindle assembly checkpoint.

Simonetta and colleagues circumvented this limitation by analysing through *in vitro* measurements and modelling a simplified spindle assembly checkpoint signalling system that includes a few basic reactions (Simonetta *et al*, 2009). Using known rate constants and concentrations, they could measure the extent of the catalytic process whereby the spindle assembly checkpoint catalyses the inhibition of Cdc20 (i.e., favours the formation of the Mad2:Cdc20 complexes). Moreover, they demonstrated the existence of the autocatalytic positive feedback loop hypothesized by the Mad2-template model (De Antoni *et al*, 2005). The loop includes the indirect inhibition model of Doncic *et al* (Figure 3B) supplemented with an autocatalytic loop (Figure 3C). Given the extremely simplified system used in this study, it is perhaps not surprising that they measured catalytic rates of Mad2:Cdc20 production that were not large enough to account for the observed dynamics of spindle assembly checkpoint activation (Simonetta *et al*, 2009).

Detailed models, including a much larger section of the spindle assembly checkpoint network acting *in vivo*, have also been developed by Ibrahim *et al* (2008a, b, 2009). Because of the lack of knowledge about the molecular mechanisms by which unattached kinetochores impinge on the spindle assembly checkpoint network, the authors represent the action of kinetochores with *ad hoc* mathematical formalisms that hinder the interpretation of biological data in terms of model's results. As such, this work provides a study in parameters that may recapitulate dynamics of spindle assembly checkpoint signalling albeit in an artificial framework.

We expect a stronger role of molecular models in the time to come when more components of the spindle assembly checkpoint network will be known in greater detail. Then it will be possible to exploit the potential of molecular models to predict new experimental results, something that is still largely unexplored. For this to happen, more data are needed.

Data are needed!

Despite the large mass of quantitative data known about the spindle assembly checkpoint, we have seen that the models developed so far have had a limited impact because of the lack of specific experimental data. In the following, we will summarize some of the measurements that would greatly aid the development of meaningful models, some of them already mentioned throughout the text.

Mad2 activation mechanisms

The mechanisms of Mad2 activation and binding to Cdc20 have yet to be fully clarified. The conversion of Mad2 from an inactive to an active form occurs at the kinetochore, catalysed by Mad1:Closed-Mad2, and possibly in the cytoplasm, catalysed by Cdc20:Closed-Mad2. The rate of catalysis measured *in vitro* was found to be too weak compared with the kinetics of the checkpoint *in vivo* (Simonetta *et al*, 2009), indicating the requirement for other molecular mechanisms at work. Besides their experimental identification, it would be desirable to repeat similar measurements if not *in vivo* at least in solution as the measurements *in vitro* were performed on reactions occurring on a surface.

Levels of Cdc20 protein during mitosis

Another element that greatly constrains the modelling work is the minimal level of free Cdc20 that is tolerated by cells without undergoing the metaphase-to-anaphase transition. Models often assume that this level is high requiring potent and complete sequestration or inhibition. Moreover, mechanisms to degrade and synthesize Cdc20 can change its concentration even during mitosis making evaluation of proposed quantitative models difficult (Nilsson *et al*, 2008). It is obvious that these assumptions have a great impact on the predictions of mathematical models and as such we need detailed measurements of Cdc20 levels during the activation and resolution of the spindle assembly checkpoint.

Release of inhibition

The molecular mechanism that underlies the disengagement of the spindle assembly checkpoint has only recently been explored. Currently, many reports agreed that Cdc20 ubiquitination has a key function in the process, but whether this involves release of inhibition remains in dispute (Pan and Chen, 2004; Reddy *et al*, 2007; Stegmeier *et al*, 2007; Nilsson *et al*, 2008). In addition, the possibility that the kinetochore itself may regulate this rate is also intriguing and worth testing (see Box 1 for a thorough discussion). Whatever the molecular mechanism, any model of the spindle assembly checkpoint cannot be formulated without a knowledge of the quantitative rate of dissociation that must match up against inhibitor production.

Kinetochore activity

The kinetochore production flux calculated in Sear and Howard (2006) is based on the number of a few species of spindle assembly checkpoint molecules localized at the kinetochore. Without an estimate of the actual number of all other spindle assembly checkpoint effectors localized at the unattached kinetochores, it will be impossible to measure the flux of all proteins that may leave the kinetochore. In addition to spindle assembly checkpoint components of presumably known function, we also require a better understanding of the role of other spindle assembly checkpoint proteins, especially the myriad of kinetochore-resident kinases.

Spindle assembly checkpoint robustness

No systems analysis of the spindle assembly checkpoint is complete without an assessment of its robustness. Intuition suggests that the capability of cells to detect even one single unattached kinetochore is likely to be robust to typical fluctuations in the concentrations of the spindle assembly

checkpoint players. An experimental estimate of this robustness has never been measured, though it is necessary for a better understanding of the wiring of the spindle assembly checkpoint network. A theoretical evaluation was reported by Doncic and collaborators, who came to the conclusion that if the spindle assembly checkpoint worked through Cdc20 sequestration it would be more robust to concentration fluctuations that may occur during checkpoint activity as opposed to a spindle assembly checkpoint that operated through Cdc20 degradation (Doncic *et al*, 2006). An experimental counterpart of this analysis, or robustness to other checkpoint protein levels, has yet to be reported.

Observations of cellular dynamics during the spindle assembly checkpoint

Direct measurements of protein dynamics and protein interactions have provided observations that inform molecular mechanisms. In addition to these experiments, there are a number of cytological observations that offer important insight into the underlying mechanisms for spindle assembly checkpoint signalling but for which an underlying molecular or quantitative basis does not yet exist. These data serve as important tests for new models under consideration.

Establishment of spindle assembly checkpoint activity
Much of the modelling efforts have focused on the last remaining unattached kinetochore and its ability to inhibit the onset of anaphase. Studies regarding the establishment of the checkpoint demonstrate a dichotomy in early signalling in which proteins such as Mad2 and BubR1, key members of the MCC complex, when depleted from cells result in a significantly shorter mitosis and increased number of mis-segregated chromosomes in comparison to other kinetochore bound proteins such as Mad1 or Bub3 (Meraldi *et al*, 2004). Importantly, this role of Mad2 and BubR1 seems to be kinetochore independent (Poddar *et al*, 2005). Although a number of hypotheses posit the role of Emi1-mediated sequestration of Cdc20 (Reimann *et al*, 2001) or Cdc20 phosphorylation (Chung and Chen, 2003; Tang *et al*, 2004) or Cyclin A (den Elzen and Pines, 2001) as early inhibitors of checkpoint activation, the sensitivity of checkpoint signalling to Mad2 and BubR1 may belie a novel pathway that is active early in mitosis.

Role of tension
Bipolar attachments are required for checkpoint silencing, consistent with the requirement that sister chromatids be segregated to opposite poles and each daughter cell receive a full complement of chromosomes. How bipolarity is sensed remains poorly understood; however, the tension generated between sister (inter-) kinetochores has been widely used as a surrogate and a potential signalling mechanism (Gorbsky and Ricketts, 1993; Nicklas *et al*, 1995). Moreover, tension is thought to regulate the activity of Aurora B that itself can regulate the stability of microtubule attachment (King and Nicklas, 2000; Cimini *et al*, 2006; Liu *et al*, 2009), the activity of the Ndc80 complex (Cheeseman *et al*, 2006; DeLuca *et al*, 2006), the recruitment of the RZZ complex (Famulski and Chan, 2007), BubR1 and Mad2 (Ditchfield *et al*, 2003), placing it at the intersection of tension and spindle assembly checkpoint signalling. This tension has recently been measured in detail in both human and *Drosophila* cells and highlights the role of intra-kinetochore tension and its impact on the spindle assembly checkpoint (Maresca and Salmon, 2009; Uchida *et al*, 2009; Wan *et al*, 2009). Together, these studies highlight an emerging molecular and quantitative understanding of attachment, tension and regulation of spindle assembly checkpoint activity. Combining existing modelling efforts in checkpoint signalling and chromosome movements (e.g. Liu *et al*, 2007 and reviewed in Mogilner *et al*, 2006) can pave the way for multi-scale models linking molecular scale motions at the kinetochore to protein diffusion and chromosome motions across the entire cell.

Positive feedback in checkpoint exit
The role of positive feedback mechanisms has been highlighted in a number of cell cycle transitions (Novak *et al*, 2007). A positive feedback in the metaphase-to-anaphase transition could provide the dynamics required for the rapid release of inhibition observed in cells, and could mirror the inherent irreversibility of sister chromatids separation. Thus far, however, no such loop has been observed. Recent work by Holt and colleagues has demonstrated the existence of a positive feedback loop that permits the rapid and switch-like activation of separase activity permitting the synchronous segregation of sister chromatids (Holt *et al*, 2008). Notably, it does not control the release of APC/C inhibition.

Experimental data related to the presence of a positive feedback loop at the metaphase-to-anaphase transition are contrasting. In budding yeast, anaphase deactivation of the checkpoint prevents its reactivation after chromosome segregation. This result has been interpreted invoking the presence of a positive feedback loop to dismantle the checkpoint through an antagonism between Mps1 and APC/C (Palframan *et al*, 2006). In mammalian cells, the silencing of the spindle assembly checkpoint is apparently reversible, to an extent, as Cyclin B degradation can be stopped by treating cells with spindle poisons after all kinetochores have attached (Clute and Pines, 1999). The widely held view of a 'point of no return' from which loss of kinetochore attachment would not result in spindle checkpoint signalling has yet to be determined quantitatively.

Diffusion of spindle checkpoint complexes
A critical assumption of many of the computational studies is the free diffusion of complexes generated from the unattached kinetochore throughout the volume of the cell. A seminal experiment that puts this assumption in dispute is the observation of fused cells in which two separate spindles undergo mitosis (Rieder *et al*, 1997). In these cells, one spindle can initiate anaphase even when the other spindle has unattached kinetochores. Once anaphase is initiated in one spindle, anaphase begins in the other even in the presence of unattached kinetochores. In principle, the spindle with unattached kinetochores should signal and prevent anaphase onset in both spindles if the diffusion of the inhibitory complex is unhindered throughout the cell. In fact, using the measurements from mitotic cells, one can estimate that the concentration of inhibitory signal (MCC or Mad2:Cdc20) should persist at least $75 \, \mu m$ from an unattached kinetochore ($\sqrt{(D/k_{diss})}$, $D \sim 10 \, \mu m^2/s$, $k_{diss} \sim 0.0017/s$). Thus, the inability to prevent anaphase onset in the fused cells has been interpreted as a diffusion barrier that keeps the

inhibitory complexes close to the spindle. If this is the case, this barrier is quite selective as it keeps the inhibitory complexes close to the spindle but permits the anaphase activating factors to diffuse from the anaphase spindle to the one with unattached kinetochores. Recent work has localized Mad2 to a spindle-like structure, termed the spindle matrix, providing a mechanism to localize the inhibitor (Lince-Faria *et al*, 2009). Sear and Howard, in their computational work, also address this observation and propose a mechanism by which the inhibitory signal is transported along spindle microtubules keeping the complexes close to the spindle surface (Sear and Howard, 2006). In either model, there is no evidence presented that the target of the checkpoint, either Cdc20 or APC/C, is similarly localized—a key point of verification for the hindered diffusion barrier hypothesis. Further work will be required to understand the nature of the original observation and the potential role of diffusion barriers in checkpoint signalling.

Conclusions and future directions

The spindle assembly checkpoint remains an exciting challenge in understanding quantitative elements of cellular signalling. In few other cellular processes is the quantitative mechanism so strongly tied to a potentially deleterious outcome. Yet the spindle assembly checkpoint is deceptively simple: produce signal, inhibit activity, attach then turn off signal. What is now apparent is that the spindle assembly checkpoint has multiple mechanisms that act together to provide the observed dynamics. Systems viewpoints can act to simplify these mechanisms, to reveal their underlying logic and deficiencies in our understanding. Further experimentation will be needed, however, to fill in the gaps in our mechanistic understanding. Achieving a fully quantitative picture will only be possible through tight interactions between experimentalists and modellers driving each other to map systems-level properties to detailed quantitative molecular mechanisms.

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