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The spindle checkpoint: a quality control mechanism which ensures accurate chromosome segregation

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Abstract

The centromere defines where on a chromosome the kinetochores assemble. Kinetochores, large protein structures, mediate chromosome segregation during mitosis and meiosis by performing three key functions. Firstly, kinetochores attach chromosomes to the microtubule spindle apparatus. Secondly, kinetochores co-ordinate microtubule dynamics to allow chromosomes to move along the spindle. Lastly, kinetochores generate the 'wait' signal which prevents anaphase onset until all the chromosomes are correctly aligned on the spindle. This signal forms part of the spindle checkpoint mechanism, a highly conserved cell cycle checkpoint which maintains the accuracy of the chromosome segregation process. This article provides a brief historical overview before focusing on some of the outstanding issues and more recent developments in the field.

Is the spindle checkpoint necessary?

Chromosome segregation is mediated by a bipolar microtubule apparatus. The bipolarity of the spindle is defined by two microtubule organizing centres (MTOCs) namely centrosomes in vertebrates and spindle pole bodies (SPB) in yeast. Microtubules are nucleated by the MTOCs and are highly dynamic structures: within a population they can exist in either growing or shrinking states and they can rapidly switch between these two states. This dynamic instability facilitates a 'search and capture' mechanism, allowing microtubules to efficiently probe the three-dimensional space around the MTOC. When a microtubule encounters a kinetochore, it becomes stabilized or 'captured', tethering the chromosome to that pole. When the sister kinetochore is captured by microtubules emanating from the opposite pole, the now bioriented chromosome congresses to the metaphase plate. The drawback of this search and capture mechanism is that the time it takes to align all the chromosomes on the metaphase plate is highly variable from one cell division to the next (Rieder *et al.* 1994). This poses a problem for the cell because the onset of anaphase is a global event: at the metaphase to anaphase transition, all the sister chromatids separate at exactly the same time (Nasmyth 2002). Therefore, any chromosome that is not bioriented when the cell commits to anaphase may not be segregated accurately, risking the production of aneuploid daughter cells (Nicklas 1997). Consequently, in order to maintain genome stability, anaphase must be delayed until all the chromosomes are

correctly bioriented. Indeed, the spindle checkpoint has evolved to do just that.

The checkpoint is essential in mammals: homozygous mutations of spindle checkpoint genes in mice results in embryonic lethality due to chronic chromosome missegregation (Dobles *et al.* 2000, Kalitsis *et al.* 2000). The checkpoint is also required for viability in *C. elegans* embryos: recovery following anoxia-induced suspended animation is dependent on several spindle checkpoint genes (Nystul *et al.* 2003). The spindle checkpoint also operates during mammalian somatic cell cycles to restrain mitotic progression (Taylor & McKeon 1997, Gorbsky *et al.* 1998). The checkpoint is not, however, essential in all organisms. Budding yeast checkpoint mutants are viable despite elevated chromosome loss rates (Li & Murray 1991, Hoyt *et al.* 1991). This may be because, in budding yeast, the kinetochores attach to the unduplicated SPB in G1. Because centromeres replicate before SPB duplication, these cells then enter mitosis with both kinetochores already attached to the old SPB (Tanaka *et al.* 2002). While these syntelic arrangements need to then be resolved to yield bioriented chromosomes, it appears that events monitored by the checkpoint are not rate limiting when yeasts are grown under optimal conditions. The checkpoint does not operate in the early *Xenopus* embryo. This is not because checkpoint components are absent but rather because the enormous cytoplasm swamps out the tiny nuclear signal (Minshull *et al.* 1994). However, because of the large cytoplasmic/nuclear ratio, *Xenopus* embryos do not employ the 'search and capture' model. The chromatin rather than MTOCs directs spindle assembly (Karsenti & Vernos 2001) and this mechanism is clearly accurate enough. (Note that chromatin may also play a more general role in directing spindle assembly; see for example Li & Zheng 2004 and references therein.) Indeed, the need to rapidly develop into a tadpole probably outweighs the risk of occasional missegregation events. It is therefore likely that the checkpoint does not offer an evolutionary advantage during early *Xenopus* development. Despite these organismal differences, research in the last decade has shown that the spindle checkpoint mechanism is highly conserved in all eukaryotes. Indeed, analysis of budding and fission yeast, worms, flies, *Xenopus* egg extracts and mammalian cells has produced a map describing how the checkpoint operates at the molecular level including identification of the sensor, the signalling pathway and the downstream effector (Amon 1999, Musacchio & Hardwick 2002, Cleveland *et al.* 2003, Lew & Burke 2003).

What does the spindle checkpoint sense?

It is now well appreciated that kinetochores play a key role in regulating the spindle checkpoint. However, what exactly is monitored remains unclear. McIntosh suggested that tension sensitive enzymes at the kinetochores generate diffusible negative regulators of anaphase (McIntosh 1991). According to this model, prior to biorientation, the lack of tension prevents anaphase. However, following biorientation, tension due to opposing spindle forces inactivates these enzymes triggering anaphase. This model had two appealing attributes. Firstly, the presence of tension means that a chromosome must be bioriented (Dewar *et al.* 2004). Secondly, by suggesting that unaligned chromosomes generate negative signals, it explained how a cell could detect a single unaligned chromosome amongst many bioriented chromosomes. Evidence for this model came from analysis of mantid spermatocytes. During meiosis I, the sex chromosomes occasionally form an XY bivalent and an unpaired X chromosome rather than an XXY trivalent. This arrangement prevents anaphase and eventually the cell degenerates. However, when a micromanipulation needle was used to artificially induce tension on the unpaired X, anaphase I initiated in a timely manner (Li & Nicklas 1995). However, the model is complicated by the fact that tension also stabilizes microtubule attachment (Nicklas & Koch 1969). Therefore, the checkpoint may be regulated by microtubule occupancy rather than tension. Indeed, in vertebrate somatic cells there is compelling evidence that microtubule attachment is the key. In PtK cells, anaphase initiates about 23 min after the last kinetochore attaches microtubules (Rieder *et al.* 1994). Furthermore, and crucially, when the last unattached kinetochore was destroyed with a laser, anaphase initiated on time (Rieder *et al.* 1995). Not only does this demonstrate that the 'anaphase wait' signal is

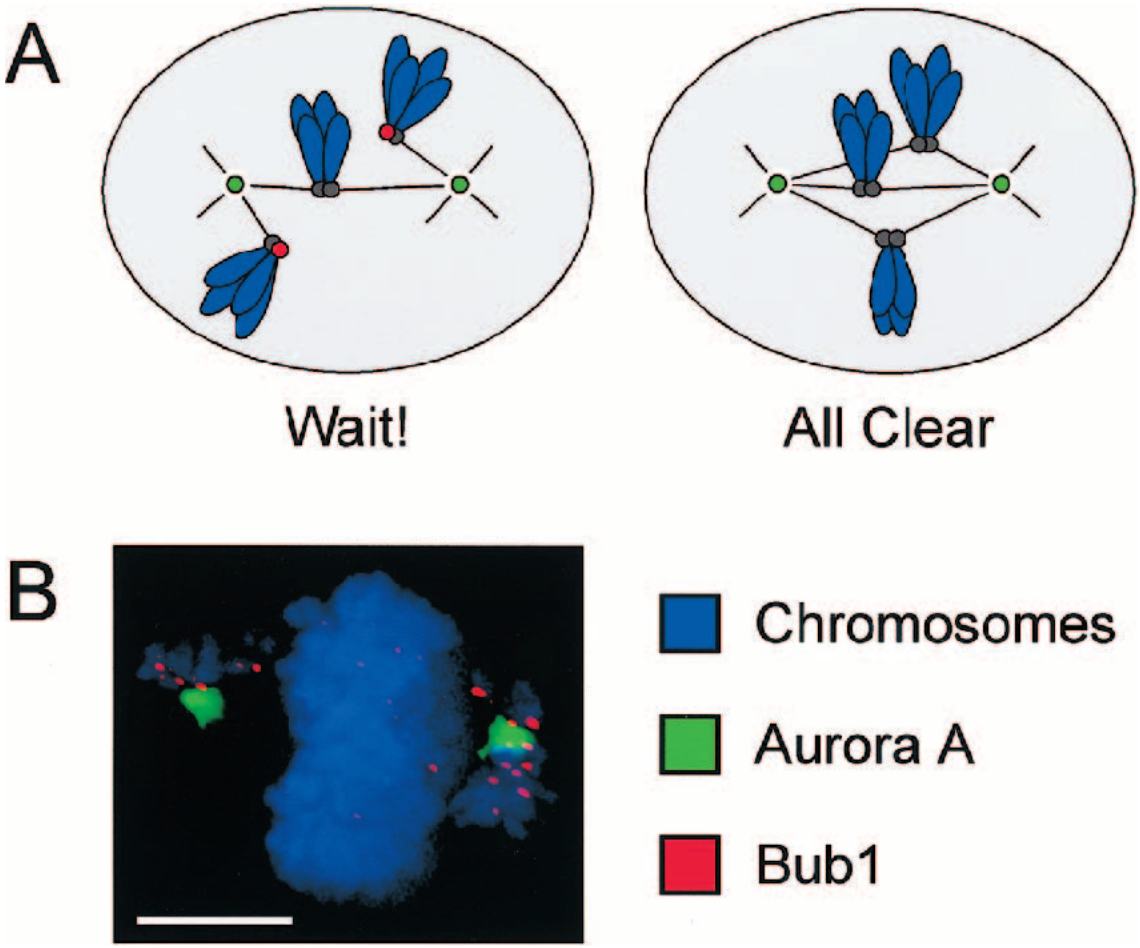

Figure 1. Unattached kinetochores activate the spindle checkpoint. (**A**) Schematic representation of a cell before (left) and after (right) all the chromosomes have become bioriented. The chromosomes are shown in blue, the spindle poles in green, microtubules as black lines. Kinetochores that are attached to microtubules are grey while unattached kinetochores are red. Prior to biorientation, the unattached kinetochores generate a negative 'Wait!' signal which prevents anaphase onset. After biorientation, the attached kinetochores no longer generate inhibitory signals, thus giving the 'All Clear' for anaphase to initiate. (**B**) A human cell stained to detect the chromosomes (blue), Aurora A (green) and Bub1 (red). The majority of the chromosomes are bioriented and lined up on the metaphase plate. The kinetochores of these chromosomes stain weakly for Bub1. A few mono-oriented chromosomes are clustered around the two spindle poles and the kinetochores of these chromosomes stain strongly for Bub1. Scale bar represents 10 μm.

generated by kinetochores (Figure 1A) but, because the remaining functional kinetochore on this mono-oriented chromosome was attached but not under tension, it indicates that microtubule occupancy, rather than tension, satisfies the checkpoint. While these differences may reflect a mitotic versus meiotic phenomenon (note that in meiosis I, sister kinetochores must co-operate and attach to the same pole whereas in meiosis II and mitosis, sisters must attach opposite poles), the prevailing view is that microtubule occupancy is the key to checkpoint silencing in somatic cells (Cleveland *et al.* 2003). However, recent analysis of Ipl1/Aurora suggests that tension cannot be ruled out (see below).

Yeast genetics identifies components of the spindle checkpoint signalling pathway

It has been appreciated for many years that anaphase is not normally initiated until all the

chromosomes are aligned on the metaphase plate (Callan & Jacobs 1957, Zirkle 1970). In addition, it has been known for over 100 years that the spindle toxin colcemid can arrest cells in mitosis. However, whether this was due to a surveillance mechanism rather than a substrate–product relationship was not clear (Hartwell & Weinert 1989). Evidence that mitotic progression was subject to checkpoint control came with the identification of budding yeast mutants that did not remain arrested in mitosis when the spindle was destroyed (Li & Murray 1991, Hoyt *et al.* 1991). Two genetic screens identified three mitotic arrest deficient mutants, *MAD1-3*, and two budding uninhibited by benzimidazole[1] mutants, *BUB1-2*. *BUB3* was then cloned as a suppressor of the *bub1-1* allele (Hoyt et al. 1991). Because microtubule depolymerization has many effects on the cell, it was initially unclear which defect(s) triggered the Mad/Bub-dependent mitotic arrest. It is now clear that, while Bub2 delays mitotic exit until the spindle enters the bud neck, Mad1/2/3 and Bub1/3 delay anaphase until all the chromosomes are bioriented. A number of other spindle checkpoint components have now been identified (Table 1).

Spindle checkpoint components localize to kinetochores

Clues as to the lesions monitored by Mad1/2/3 and Bub1/3 proteins came with the identification of their vertebrate counterparts which demonstrated that all these proteins localize to kinetochores in mitosis (Li & Benezra 1996, Chen *et al.* 1996, Taylor & McKeon 1997, Taylor *et al.* 1998, Chen *et al.* 1998). Note that while budding and fission yeast express one Bub1 and one Mad3 protein, vertebrates express two Bub1-related protein kinases, Bub1 and BubR1 (Taylor *et al.* 1998). BubR1 is related to both Bub1 and Mad3 (Figure 2A); hence some database entries refer to it as Mad3L or Bub1b. All these proteins localize to kinetochores during the early phases of mitosis but are less abundant following chromosome alignment (Figure 1B). Thus, the Mad and Bub proteins are in the right place at the right time to monitor kinetochore–microtubule interactions, consistent with playing a role in the mechanism which delays anaphase until all the chromosomes biorient. Although the Mad and Bub proteins dissociate from kinetochores prior to anaphase, dissociation is not a prerequisite for anaphase. If the checkpoint is overridden with a dominant negative Mad1 mutant or by repression of BubR1, cells undergo a premature anaphase with Mad2 and Bub1 still bound to kinetochores (Canman *et al.* 2002, Ditchfield *et al.* 2003). Clearly therefore, kinetochore-bound Bub1 and Mad2 is not sufficient to prevent anaphase. More recent evidence even suggests that efficient kinetochore localization of these checkpoint proteins may not be essential for checkpoint activation. For example, in the presence of Aurora kinase inhibitors, kinetochore localization of BubR1, Mad2 and CENP-E is severely compromised yet the cells still arrest in mitosis when the spindle is destroyed (Ditchfield *et al.* 2003). In addition, when a component of the Ndc80 complex, Hec1, is repressed, Mad1, Mad2 and Mps1 are not detected at kinetochores yet the cells arrest (Martin-Lluesma *et al.* 2002). These observations appear to raise doubts about the importance of kinetochore localization. However, the following three points must be kept in mind. Firstly, localization does not equate to function: the presence of checkpoint components at kinetochores does not necessarily mean they are active. Likewise, the low abundance of checkpoint proteins at kinetochores during a prometaphase arrest does not rule out the possibility that they were first activated in a kinetochore-dependent manner but can sustain their activity in the cytoplasm, independent of the kinetochore (DeLuca *et al.* 2003). Secondly, it is not certain that kinetochore localization is the rate-limiting step in checkpoint activation/deactivation. Indeed, FRAP[2] analysis indicates that the checkpoint proteins flux rapidly through

[1]In the presence of the microtubule depolymerization agent benzimidazole, wild-type yeast cells arrest in mitosis as large budded cells. However, because the Bub mutants cannot maintain the mitotic arrest, they return to interphase without dividing then commit to a new cell cycle, forming a new bud.

[2]Fluorescence recovery after photobleaching.

Table 1. Components of the spindle checkpoint and associated functions.

Component	Proposed role in the regulation of anaphase onset
Bone fide checkpoint components	
Mad1	Coiled-coil protein, localizes to kinetochores during mitosis and recruits Mad2. *In vitro* substrate for Bub1 and Mps1. Phosphorylated upon checkpoint activation in yeast.
Mad2	Localizes to unattached kinetochores through association with Mad1. Binds to Cdc20 and inhibits the APC/C *in vitro*. Forms part of the mitotic checkpoint complex (MCC).
Mad3	Yeast Mad3 is similar to BubR1 but lacks the kinase domain. Binds to Bub3, Mad2 and Cdc20 and forms part of the MCC.
Bub1	Protein kinase which localizes to kinetochores very early in prophase in a Bub3-dependent manner. Subsequently recruits other checkpoint proteins to the kinetochore.
Bub3	Contains 4 WD repeats, targets Bub1 and BubR1 to kinetochores and is part of the MCC. Very similar to Rae1, a protein involved in nucleo-cytoplasmic transport.
BubR1	BubR1 is similar to Mad3 yet contains a C-terminal kinase domain which is stimulated by CENP-E. BubR1 is required both as an enzyme and a stoichiometric inhibitor. A component of the MCC.
Mps1	Also known as Mph1 and TKK. Mps1 is a dual specificity protein kinase required for centrosome duplication and spindle checkpoint function. Phosphorylates Mad1 *in vitro*.
Kinetochore proteins linked to checkpoint function	
Ndc80	The Ndc80 complex, consisting of Ndc80/Hec1, Nuf2, Spc24 and Spc25, is required to recruit a subset of checkpoint proteins to the kinetochore.
Rod/ZW10	The Zeste-white10 and Rod complex is required for checkpoint function in flies and human cells. It is not required for Bub1/3 recruitment. Relationship with other checkpoint proteins remains unclear.
Downstream effectors of the checkpoint	
Cdc20	Also known as Slp1, Fizzy (FZY) and p55Cdc. Contains 7 WD repeats, activates the APC/C, targeting Securin and the mitotic cyclins for degradation. Downstream target of the checkpoint.
Cdh1	Also known as Hct1 and Fizzy-related (FZR). In budding yeast Cdh1 is downstream of the mitotic exit network. In higher eukaryotes Cdh1 maintains proteolysis in G1.
APC/C	The anaphase promoting complex or cyclosome is a multiprotein complex with E3 ubiquitin ligase activity.
Downstream effectors of the APC/C	
Securin	Also known as Pds1 in budding yeast, Cut2 in fission yeast, and PTTG in humans. An anaphase inhibitor which is targeted for degradation by APC/C-Cdc20. Binds and thus inhibits Separase.
Separase	Also known as Esp1 in budding yeast, and Cut1 in fission yeast. A protease that cleaves Cohesin thus triggering sister chromatid separation. Prior to anaphase sequestered by Securin.
Cohesin	A multiprotein complex consisting of Scc1 and two large ATPases, Smc1 and Smc3. Forms a ring structure which holds sister chromatids together. Cleavage of Scc1 triggers sister chromatid separation.
The Ipl1/Aurora family	
Ipl1	Sole member of the Ipl1/Aurora family present in budding yeast. A kinetochore component, which regulates microtubule binding and checkpoint function.
ARK1	The fission yeast Aurora kinase homologue, required for chromosome condensation and the kinetochore attachment checkpoint response.
Aurora A	Localizes to centrosomes/spindle poles, is implicated in centrosome maturation, mitotic entry and pole separation. Overexpression linked to tumour evolution and spindle checkpoint dysfunction.
Aurora B	A chromosome passenger protein, which binds INCENP (Sli15) and Survivin (Bir1). Implicated in phosphorylation of histone H3, chromosome segregation and cytokinesis.
Aurora C	Mammals express a third Aurora kinase. Abundantly expressed in testis and localizes to the spindle pole but little else known.
Other players	
APC	Adenomatous polyposis coli protein, acts as a tumour suppressor function in Wnt signalling pathway. Localizes to microtubules and kinetochores during mitosis where it may regulate kinetochore–microtubule interactions.
MAPK	Implicated in the spindle checkpoint but precise role unclear. Required for checkpoint arrest in *Xenopus* egg extracts and phosphorylates Cdc20.
CMT2	Binds Mad2 and may play a role in down regulating the checkpoint signal in late mitosis (Habu *et al.* 2002).

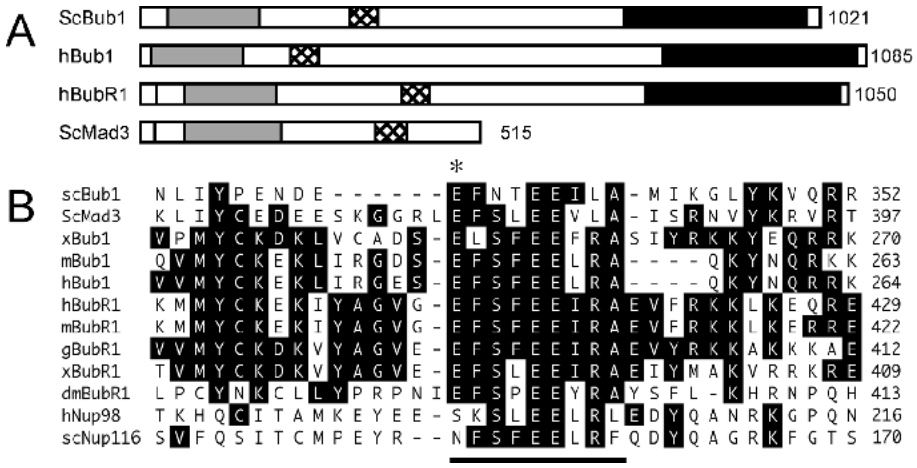

Figure 2. Higher eukaryotes express two Bub1-related protein kinases, Bub1 and BubR1. (**A**) Schematic representation of Bub1 and Mad3 related proteins from budding yeast and humans showing the N-terminal homology domains (grey shading), the C-terminal kinase domains (black shading) and the Bub3 binding/kinetochore localization domain (hatched box). The vertical line in BubR1 and Mad3 represents a KEN sequence. Note that, although BubR1 has a kinase domain, in the N-terminus it is more similar to Mad3 than Bub1. (**B**) Sequence alignments of the Bub3 binding/kinetochore localization domain from a variety of Bub1/BubR1-related proteins. The horizontal line shows the EFSFEEIRA highly conserved core consensus sequence while the * marks the glutamic acid which is mutated to a serine in the *bub1-1* allele. The GLEBS motifs from Nup98 and Nup116 are also shown. Note that, although similar to the Bub3-binding site in Bub1, the conserved glutamic acid is not present in the GLEBS motif.

the kinetochore, with the half-life of Mad2 recovery being of the order of 24 s (Howell *et al.* 2000). Therefore, perhaps only small amounts of checkpoint proteins need to interact with unattached kinetochores in order to sustain the checkpoint. Finally, if the kinetochore is completely ablated in budding yeast by mutation of the Cbf3 components, the cells undergo anaphase and exit mitosis despite chromosomes failing to attach to the spindle (Gardner *et al.* 2001, Goh & Kilmartin, 1993).

The APC/C is the downstream effector of the checkpoint

While our understanding of how kinetochores activate/deactivate the checkpoint remains vague, it is clear that the downstream effector of the checkpoint is an E3 ubiquitin ligase called the anaphase-promoting complex or cyclosome (APC/C; see Figure 3). Prior to anaphase, the replicated sister chromatids are held together by a protein complex known as Cohesin. This ring-shaped structure encircles the chromatids and needs to be cleaved in order to liberate the two sisters (Gruber *et al.* 2003). While a single cleavage anywhere in the ring will suffice, in normal cells it is the small subunit Scc1 that is cleaved and this is done by a protease known as Separase (Uhlmann *et al.* 1999, 2000). This protease is normally bound to an inhibitor known as Securin and therefore, in order for Separase to become activated, it must be released from Securin (Ciosk *et al.* 1998). This is achieved by 26S proteosome-mediated destruction of Securin. Securin is targeted for degradation by polyubiquitination, a reaction that is catalysed by the ubiquitin ligase activity of the APC/C. The APC/C also targets mitotic cyclins for degradation, thus triggering mitotic exit. While the details of the ubiquitination step remain to be solved, it is clear that the key regulatory step is the activation of the APC/C (Peters 2002, Murray 2004). Two APC/C activators have been identified, Cdc20 and Cdh1. These two structurally related proteins target different substrates for proteolysis and therefore also appear to be 'specificity factors'. In vertebrates, Cdc20 activates APC/C-mediated destruction of Securin and the mitotic cyclins. Importantly, the spindle checkpoint prevents anaphase by inhibiting Cdc20-mediated activation of the APC/C.

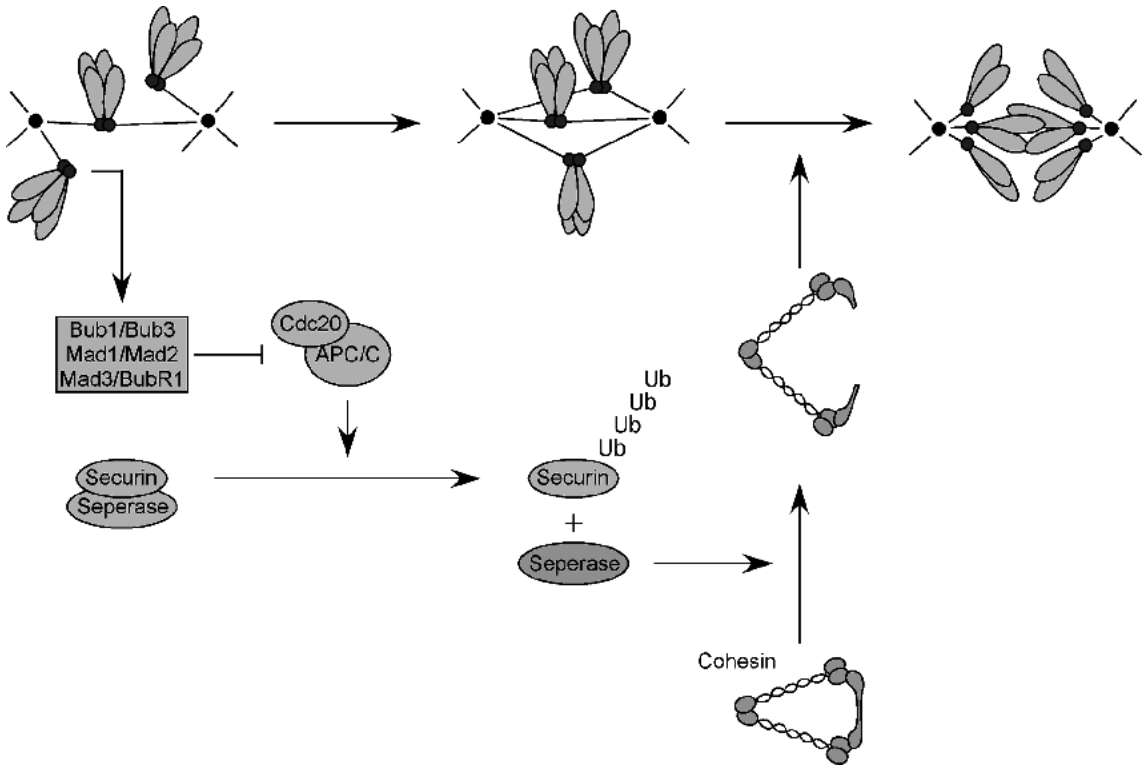

Figure 3. The APC/C is the downstream target of the spindle checkpoint. Unattached kinetochores activate the Mad/Bub-dependent checkpoint pathway which inhibits Cdc20-mediated activation of the APC/C. When all the chromosomes align, the checkpoint signal is extinguished thus allowing Cdc20 to activate the APC/C. Securin is then polyubiquitinated, targeting it for proteolysis and thus releasing Separase. Separase then cleaves the Scc1 subunit of Cohesin, opening the ring structure and thus allowing sister chromatids to separate.

A major advance came with the demonstration that Mad2 interacts with Cdc20 (Hwang *et al.* 1998, Kim *et al.* 1998). Yeast cells harbouring Cdc20 mutants that can not bind Mad2 fail to activate the checkpoint. In *Xenopus* egg extracts, Mad2 binds to APC/C thus preventing it from ubiquitinating Cyclin B (Fang *et al.* 1998). When expressed in bacteria, Mad2 readily forms tetramers which inhibit APC/C more effectively than Mad2 monomers, suggesting that the role of the kinetochore is to convert Mad2 into a form that can inhibit the APC/C, possibly by sequestering Cdc20 (Shah & Cleveland 2000). This model is probably an oversimplification: tetrameric forms of Mad2 have not been identified in cells or extracts. Rather, in HeLa cells, a subpool of Mad2 is part of the mitotic checkpoint complex (MCC), which also includes BubR1, Bub3 and Cdc20 (Sudakin *et al.* 2001). This complex is a very potent inhibitor of APC/C. Indeed, while recombinant Mad2 and BubR1 can separately inhibit the APC/C, together they exhibit a synergistic effect (Tang *et al.* 2001, Fang 2002). Thus, the MCC is an attractive candidate for the physiologically relevant APC/C inhibitor. Indeed, a complex consisting of Mad2, Mad3, Bub3 and Cdc20 has been identified in yeast (Hardwick *et al.* 2000, Fraschini *et al.* 2001, Millband & Hardwick 2002). A similar complex exists in metaphase *Xenopus* extracts and is enriched upon the addition of unattached kinetochores (Chen 2002). However in yeast and HeLa cells, assembly of MCC appears to be independent of kinetochores. Perhaps therefore, the role of the kinetochore is not to catalyse the formation of the MCC but rather to convert the MCC to a form that can inhibit the APC/C. Consistent with kinetochores converting the MCC to an active form, Mad1 is required to recruit Mad2 to kinetochores

(Chen *et al.* 1998). However, the binding of Mad2 to Mad1 and Cdc20 is mutually exclusive, suggesting that Mad2 may bind to Cdc20 only after it has been recruited to the kinetochore (Sironi *et al.* 2001, 2002). Furthermore, kinetochore-dependent phosphorylation of Cdc20 may be required to keep it inactive (Chung & Chen 2003). Mad2 is also phosphorylated in checkpoint activated cells yet in contrast to Cdc20, this appears to abolish its ability to activate the checkpoint (Wassmann *et al.* 2003).

Kinetochore localization of the Mad and Bub proteins is differentially regulated

Mad2 localizes to kinetochores in prometaphase but not in metaphase. Furthermore, on a mono-oriented chromosome, the attached kinetochore is negative for Mad2 whereas the unattached one stains brightly for Mad2 (Chen *et al.* 1996). In addition, if metaphase cells are exposed to taxol, a spindle toxin which stabilizes microtubules thus reducing tension without necessarily detaching the chromosomes, Mad2 is not re-recruited to kinetochores (Waters *et al.* 1998). Thus, Mad2 localization is clearly regulated by microtubule attachment. Because Mad2 is essential for checkpoint activation (Shannon *et al.* 2002), does this imply that the checkpoint is indeed regulated by microtubule occupancy? Not necessarily: while dampening microtubule dynamics with taxol does not detach chromosomes from the spindle, it does activate the checkpoint (Kelling *et al.* 2003). Indeed, although Mad2 is not re-recruited under these conditions, kinetochores become rephosphorylated, as judged by 3F3/2[3] reactivity (Waters *et al.* 1998). Clearly, therefore, mitotic kinetochores are sensitive to changes in tension. Does this suggest that the checkpoint is able to monitor tension? Again, not necessarily: while most kinetochores in these taxol-treated cells are Mad2 negative, at least one Mad2-positive, and therefore presumably unattached, kinetochore is always present (Waters *et al.* 1998).

In prometaphase cells, kinetochore localization of Bub1 is frequently asymmetric with the weaker kinetochore oriented towards the nearest spindle pole (Taylor *et al.* 2001). In contrast the localization of BubR1 is symmetrical. This suggests that, like Mad2, the localization of Bub1 is sensitive, at least in part, to microtubule occupancy. Consistently, in both humans and *Xenopus*, inhibition of microtubule polymerization dramatically increases the levels of kinetochore-bound Bub1 (Sharp-Baker & Chen 2001, Taylor *et al.* 2001). In contrast, the levels of kinetochore-bound BubR1 are relatively insensitive to microtubule depolymerization. Rather, reduction in kinetochore-bound BubR1 does not occur until after biorientation suggesting that it is a tension sensor (Taylor *et al.* 2001). Indeed, when tension is inhibited at metaphase kinetochores with low doses of vinblastine, BubR1 is re-recruited to kinetochores (Skoufias *et al.* 2001).

Bub1: a master regulator required for assembly of the kinetochore signalling domain

The checkpoint proteins are not recruited simultaneously to kinetochores in human cells. Rather there appears to be a defined order of assembly. Specifically, Bub1 is recruited to kinetochores very early in prophase, followed by CENP-F, BubR1, CENP-E and finally Mad2 in late prometaphase (Jablonski *et al.* 1998, Taylor *et al.* 2001, Johnson *et al.* 2004). One model to explain this temporal order of assembly is that recruitment of the latter proteins is dependent on the prior recruitment of the early ones (Figure 4). Indeed, immunodepletion of Bub1 from egg extracts prevents kinetochore localization of BubR1, Mad2, Mad1 and CENP-E (Sharp-Baker & Chen 2001). Similarly, repression of Bub1 by RNAi in human cells inhibits kinetochore localization of BubR1, Mad2, CENP-E and CENP-F. However, immunodepletion of BubR1 from extracts prevents recruitment of Bub1, Mad2, Mad1 and CENP-E (Chen 2002). This observation seems at odds with the temporal order observed in mammalian cells. Indeed, when BubR1 is repressed by RNAi in human cells, Bub1's ability to localize to kinetochores is unaffected (Johnson *et al.* 2004). Likewise CENP-F

[3]3F3/2 is a monoclonal antibody which recognizes a number of mitotic phosphoproteins, at least one of which localizes to kinetochores that are not under tension (see Nicklas 1997).

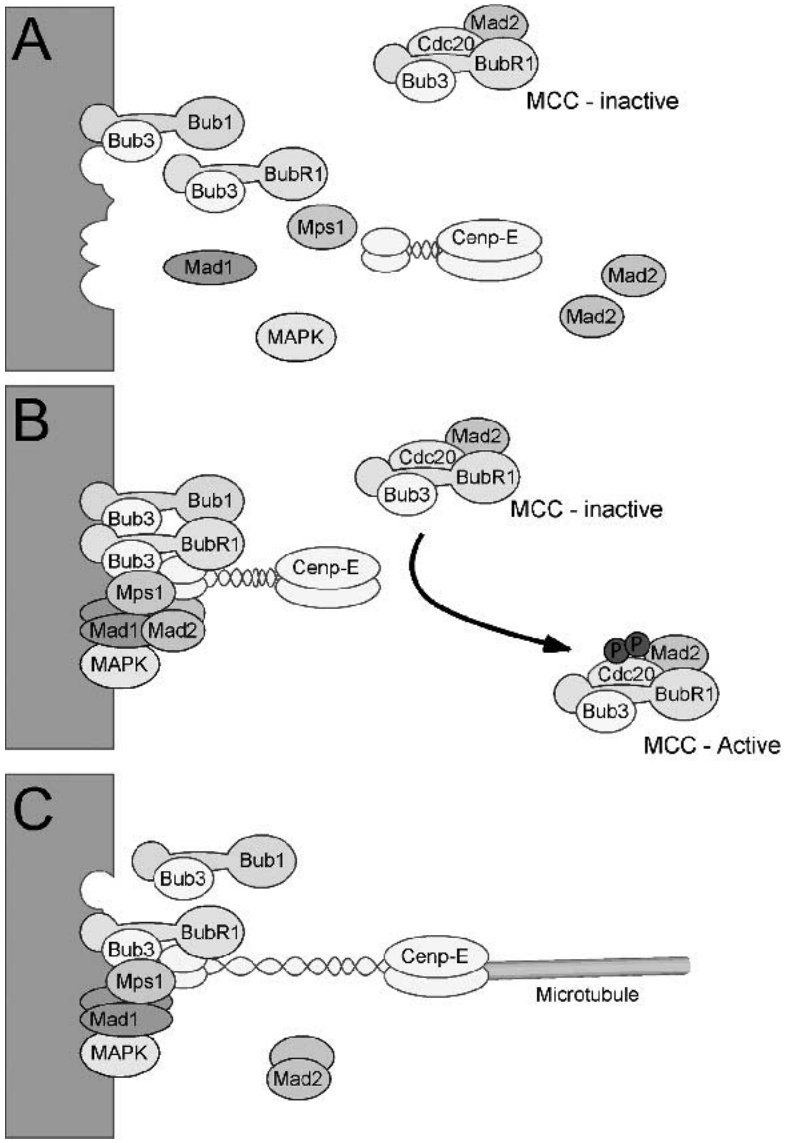

Figure 4. Model describing how unattached kinetochores generate an anaphase inhibitor. (**A**) Phase 1, assembly of active checkpoint signalling complexes. Upon entry into mitosis the various checkpoint proteins are recruited to kinetochores in a defined order. First Bub1 targets the kinetochore in a Bub3-dependent manner. This is followed by BubR1 (which also requires Bub3), Mps1, CENP-E, Mad1 and Mad2. (**B**) Phase 2, generation of the anaphase inhibitor. Upon assembly of the signalling complexes, CENP-E stimulates the kinase activity of BubR1 which somehow contributes to the generation of the anaphase inhibitor. A leading candidate for the inhibitor is the MCC, consisting of BubR1, Bub3, Mad2 and Cdc20. The relationship between MCC components that are part of the soluble MCC in contrast to those bound to the kinetochore is not clear. While there could be exchange reactions, there could also be two separate pools of these proteins. The role of kinase activity and phosphorylation events is unclear. Phosphorylation of substrates, in particular Cdc20, may be part of the mechanism converting the inhibitor from an inactive to active state. (**C**) Phase 3, down regulation of the signal. When CENP-E attaches a microtubule, the kinase activity of BubR1 is down regulated. Attachment sensors such as Mad2 and Bub1 become less abundant at the kinetochore while others, such as BubR1, remain bound until biorientation. As a consequence of either or both of these events, the anaphase inhibitor is no longer generated.

and Mad2 are unaffected following BubR1 RNAi although CENP-E fails to target the kinetochore, consistent with the fact that BubR1 and CENP-E physically interact (Chan *et al.* 1998, Yao *et al.* 2000).

Cloning of Bub1, by complementation of the *bub1-1* allele, indicated that it is a protein kinase with a C-terminal kinase domain (Roberts *et al.* 1994). Sequence comparison with the first mammalian homologue revealed that, in addition to the kinase domain, the yeast and mammalian proteins shared a block of homology in the N-terminus (Taylor & McKeon 1997). This domain (see Figure 2A) is also present in Mad3 and BubR1 (Taylor *et al.* 1998). Because Bub3 was cloned as a suppressor of the *bub1-1* allele (Hoyt *et al.* 1991), it suggested that Bub1 and Bub3 might interact. This is indeed the case in both yeast and mammals (Roberts *et al.* 1994, Taylor *et al.* 1998). Deletion mapping of Bub1 narrowed down the Bub3-binding site to within amino acids 200–300 (Figure 2B). Closer inspection of the sequence alignments identified a conserved region that is also present in Mad3 and BubR1 (Taylor *et al.* 1998). Deletion of this short $\sim$40 amino acid motif abolishes the ability of both Bub1 and BubR1 to bind Bub3. Importantly, the ability to bind Bub3 is required for both Bub1 and BubR1 to localize to kinetochores. Furthermore, a mutation in Bub3 which prevents Bub1 binding abolishes its ability to localize to kinetochores (Taylor *et al.* 1998). This suggests that in order to localize to kinetochores, Bub1 and Bub3 must be complexed together. Consistent with these observations, BubR1 fails to localize to kinetochores in a *Drosophila* Bub3 mutant and likewise Bub3 fails to localize to kinetochores in a BubR1 mutant (Basu *et al.* 1998)[4]. Bub1 and Mad3 localize to kinetochores in *S. pombe* (Bernard *et al.* 1998 Millband & Hardwick 2002) and, more recently, a very careful study using GFP-tagged proteins and chromatin immunoprecipitation has confirmed that Bub1 localizes to kinetochores in budding yeast and that this is dependent on Bub3 (Gillett *et al.* 2004). A one-hybrid assay also confirms that Bub1 localizes to kinetochores in budding yeast (Warren *et al.* 2002). Interestingly, the nature of the *bub1-1* allele has been determined. The sole change is a G to A mutation at position 997 which substitutes a glutamic acid for a lysine (E333K; Warren *et al.* 2002). This glutamic acid is highly conserved amongst the Bub1 related proteins (Figure 2B) and is in the middle of the Bub3 binding site (Taylor *et al.* 1998) explaining why overexpression of Bub3 can complement the *bub1-1* allele. The Bub1 E333K mutant does not 'band shift' suggesting that kinetochore localization and/or Bub3 binding is required for its phosphorylation. Indeed, in contrast to BubR1, phosphorylation of Bub1 appears to depend on an activated spindle checkpoint (Taylor *et al.* 2001).

The role of kinase activity in checkpoint activation

Little is known about the role of Bub1 kinase activity. In a benomyl sensitivity assay, a Bub1 kinase mutant (K733R) cannot complement the *bub1-1* allele indicating that Bub1 kinase activity is essential for checkpoint function (Roberts *et al.* 1994). Consistently, in *S. pombe*, Bub1's kinase activity appears to be required for checkpoint fuction (Yamaguchi *et al.* 2003). However, following immunodepletion of Bub1 from *Xenopus* egg extracts, checkpoint function can be restored by adding back a recombinant kinase mutant (Sharp-Baker & Chen 2001). Indeed, a more recent yeast study indicates that the N-terminal 608 amino acids of Bub1 can perform all the checkpoint functions of the wild-type protein despite completely lacking the kinase domain (Warren *et al.* 2002). How can these observations be reconciled with the earlier K733R result? The K733R mutation appears less stable than the wild type (Warren *et al.* 2002). Thus, following prolonged periods at the restrictive temperature required for the colony formation assay, the inability of the K733R mutant to complement the *bub1-1* allele may be due to the fact that the protein does not accumulate to similar levels as the wild-type protein. Indeed, in liquid culture, the K733R mutant is capable of sustaining a robust checkpoint arrest for 4 h.

[4]Note that although originally described as a *Drosophila* Bub1 homolog, sequence alignments indicate that the protein identified by Basu *et al* (1998) is more closely related to BubR1.

If Bub1's kinase activity is not required for its checkpoint function, what role does it play? Interestingly, chromosome loss rates in Bub1 and Bub3 deficient yeast strains are 2–3 times higher than those of Mad1 and Mad2 deficient strains (Warren *et al.* 2002). One possible explanation for this comes from a recent study showing that Bub1/3 localize to budding yeast kinetochores during a normal mitosis while Mad1/2 are only recruited following microtubule detachment (Gillett *et al.* 2004). Because yeast kinetochores are almost always attached to microtubules (see above), Mad1/2 would only be required in exceptional circumstances. Another possibility is that Bub1/3 perform functions in addition to their checkpoint roles. Indeed, there is evidence that Bub1 is required for chromosome congression in human cells (Johnson *et al.* 2004). Rather surprisingly, RNAi-mediated repression of Bub1 does not compromise the checkpoint in response to nocodazole but does result in ~80% of the metaphase cells having one or more chromosomes clustered near a spindle pole. Whether Bub1's role in chromosome congression requires its kinase activity remains to be seen. A further role for Bub1 has emerged from studies in fission yeast where it targets Sgo1 and Sgo2 to the centromere thus maintaining centromeric cohesion between sister chromatids during meiosis I (Kitajima *et al.* 2004).

The requirement for BubR1 kinase activity has been equally confusing. Although BubR1 contains a kinase domain, its yeast counterpart Mad3 does not (Taylor *et al.* 1998). In addition, BubR1's kinase domain is quite divergent from other kinases and that of Bub1. Indeed, the kinase domains of *S. cerevisiae* and human Bub1 are more similar to each other than are the kinase domains of human Bub1 and human BubR1. Several amino acids that are thought to be invariant in kinase domains are also not conserved in BubR1 (Taylor *et al.* 1998). Efforts in this area have been hampered because immunoprecipitated BubR1 performs weakly in *in-vitro* kinase assays. These observations clearly cast doubt on the importance of BubR1 as a kinase. Indeed, it was reported that a recombinant BubR1 kinase mutant could restore checkpoint function to an immunodepleted *Xenopus* egg extract (Chen 2002). However, a second study demonstrated that a BubR1 kinase mutant could not restore checkpoint function to a BubR1-depleted extract (Mao *et al.* 2003). One explanation for this discrepancy is that perhaps the first result arose due to incomplete immunodepletion of BubR1. In support of this, while adding back wild-type BubR1 to ~20% of its normal level did not restore the checkpoint, adding back an 80%/20% mixture of the kinase mutant and wild-type BubR1 did (Mao *et al.* 2003). This suggests that BubR1 may have two inhibitory functions, one as a catalytic inhibitor, the other as a stoichiometric inhibitor (see Figure 4). This latter role fits well with the notion that the MCC is a stoichiometric inhibitor of the APC/C, but what about the catalytic role? Like immunoprecipitated human BubR1, *Xenopus* BubR1 is also a very poor kinase *in vitro*. However, if CENP-E is added, the kinase activity of BubR1 is stimulated several fold (Mao *et al.* 2003). Furthermore, if an anti-CENP-E antibody is added, BubR1 activity is down regulated. Rather than disrupting the CENP-E–BubR1 association, this particular antibody binds the microtubule binding domain of CENP-E. Taken together with the observations that CENP-E is required for spindle checkpoint function in both egg extracts and mammalian somatic cells (Abrieu *et al.* 2000, Weaver *et al.* 2003), these observations evoke an interesting model whereby in the absence of microtubule–kinetochore interactions, CENP-E stimulates BubR1 thus activating the checkpoint. Upon CENP-E binding microtubules, BubR1 is down regulated giving the all clear for anaphase (see Figure 4).

Mps1 And MAP kinases

Although originally identified as being required for spindle pole body duplication, Mps1 is also required for spindle checkpoint function in yeast (Weiss & Winey 1996). Overexpression of Mps1 constitutively activates the checkpoint in yeast, independent of spindle damage (Hardwick *et al.* 1996, He *et al.* 1998). This arrest is dependent on the other Mad and Bub genes indicating that Mps1 plays an early role in the signalling cascade. Mps1 can phosphorylate Mad1 *in vitro* and Mad1 is also phosphorylated upon checkpoint activation in yeast (Hardwick & Murray 1995, Hardwick *et al.* 1996). This requires Mps1 as well as Bub1 and Bub3, placing Mps1, Bub1 and

Bub3 upstream of Mad1. However, Mad1 phosphorylation is not essential for checkpoint activation. The *BUB1-5* allele hyper activates the checkpoint but under these conditions Mad1 is not phosphorylated (Farr & Hoyt 1998). Homologues of Mps1 (also known as TTK) have also been studied in *Xenopus* and mammals, and in both cases they are required for spindle checkpoint function (Fisk & Winey 2001, Abrieu *et al*, Fisk *et al.* 2003). There is, however, some controversy as to whether Mps1 is required for centrosome duplication in mammals (Stucke *et al.* 2002, Fisk *et al.* 2003). These differences may reflect the different threshold levels of Mps1 that are required for its two functions (Fisk *et al.* 2003).

Early experiments with *Xenopus* egg extracts which showed that the spindle checkpoint could be reconstituted *in vitro* implicated MAP kinase (MAPK) signalling as being required for checkpoint arrest (Minshull *et al.* 1994). However, these observations are complicated by the fact that *Xenopus* egg extracts are only one or two cell cycles away from what was a meiotic cell, naturally arrested at metaphase II due to cytostatic factor (CSF), which in turn is dependent on MAPK activity (Tunquist & Maller 2003). Because stimulation of MAPK signalling can reactivate CSF in a fertilized embryo leading to mitotic arrest (Haccard *et al.* 1993), it is possible that the apparent role of MAPK in the spindle checkpoint is due to its ability to reactivate CSF. However, there is evidence that MAPK activity is required for checkpoint function in somatic cells (Wang *et al.* 1997, Takenaka *et al.* 1998). Importantly, activated ERKS (extracellular signal-regulated protein kinases) localize to kinetochores (Shapiro *et al.* 1998, Zecevic *et al.* 1998) and MAPK may be responsible, at least in part, for phosphorylation of Cdc20 (Chung & Chen 2003). Intriguingly, in *Xenopus*, it now appears that components of the spindle checkpoint play a role in mediating CSF-dependent metaphase arrest in meiosis II (Tunquist *et al.* 2003).

The Ipl1/Aurora family of protein kinases

The Ipl1 protein kinase is a kinetochore component in budding yeast and has recently been implicated in spindle checkpoint function. Ipl1 is not required to induce mitotic arrest following loss of kinetochore–microtubule interactions but is required under conditions that prevent the kinetochore from coming under tension, for example in cohesion or replication mutants (Biggins & Murray 2001). Mammalian cells, which express three Ipl1-related protein kinaes, Aurora A, B and C, exhibit a similar phenotype following exposure to two small molecule Aurora inhibitors, ZM447439 and Hesperadin (Hauf *et al.* 2003, Ditchfield *et al.* 2003). RNAi-mediated repression of Survivin, an Aurora B interactor, also yields a similar phenotype (Lens *et al.* 2003, Carvalho *et al.* 2003). Specifically, in the absence of Aurora kinase activity or Survivin, cells can arrest in mitosis when the spindle is destroyed with nocodazole but exit mitosis in the presence of taxol which allows kinetochores to bind microtubules but inhibits tension. While at first glance this suggests that the checkpoint does indeed monitor tension, these observations are complicated by the fact that Ipl1 has been shown to destabilize kinetochore–microtubule interactions that do not yield tension (Tanaka *et al.* 2002). Likewise, Aurora kinase activity is required to resolve kinetochore–microtubule interactions that do not result in a correctly bioriented chromosome (Hauf *et al.* 2003). Thus, the apparent requirement for Ipl1/Aurora in the checkpoint may be a secondary consequence of its ability to reduce microtubule occupancy at kinetochores.

However, if this was universally true, one would expect that, in the absence of Aurora kinase activity, spindle checkpoint proteins would localize to kinetochores that lacked bound microtubules. However, ZM447439 dramatically reduces the levels of kinetochore-bound BubR1 and Mad2 in nocodazole-treated cells (Ditchfield *et al.* 2003). A second explanation therefore for the nocodazole taxol difference is that, although inhibition of Aurora kinase activity reduces the amount of checkpoint proteins at the kinetochore, perhaps the residual bound protein is sufficient to sustain mitotic arrest in the absence of kinetochore–microtubule interactions. If microtubule occupancy is sufficient to inactivate the remaining bound proteins, this may explain why Aurora-deficient cells cannot arrest in the presence taxol (Ditchfield *et al.* 2003). The third

possible explanation suggests that the spindle checkpoint pathway is composed of two arms, one of which depends on Aurora kinase activity and one which does not. While there are at present no data to support this notion, it is intriguing that Bub1 repressed cells arrest in nocodazole, suggesting that, like Aurora kinase activity, Bub1 is not essential for checkpoint function (Salina *et al.* 2003, Johnson *et al.* 2004). The currently available Aurora kinase inhibitors do not discriminate between Aurora A and B. However, the currently available RNAi data (Ditchfield *et al.* 2003) plus that derived from expression of kinase mutants, injection of antibodies (Kallio *et al.* 2002, Murata-Hori & Wang 2002) and the similar phenotype observed following repression of Survivin (Carvalho *et al.* 2003, Lens *et al.* 2003) all indicate that the checkpoint defects induced by inhibition of Aurora kinase activity are due to inhibition of Aurora B.

The spindle checkpoint and nucleo-cytoplasmic transport

Bub3 contains four WD repeats and homologues have been identified in many eukaryotes. Bub3 is very closely related to another WD repeat protein, Rae1, and importantly, the similarity is not just confined to the WD repeats (Taylor *et al.* 1998). Originally identified as an RNA export mutant in *S. pombe*, Rae1 homologues have been identified in *S. cerevisiae* (Gle2) and mammals. Gle2 binds to nucleoporins, namely Nup116 in budding yeast and Nup98 in humans. The Gle2 binding site in these nucleoporins has been defined and termed the GLEBS motif (Gle2 binding sequence) (Bailer *et al.* 1998, Pritchard *et al.* 1999). Interestingly, the GLEBS motif is very similar to the domain defined in Bub1/BubR1 as being required for binding to Bub3 (Figure 2B). Indeed, Rae1 can bind Bub1 and localize to kinetochores when overexpressed (Wang *et al.* 2001). The significance of these results is unclear but strikingly $Rae1^{+/-}$ mouse embryonic fibroblasts exhibit a checkpoint defect (Babu *et al.* 2003). Whether this reflects a direct role for Rae1 in the checkpoint or whether the phenotype is due to a nucleo-cytoplasmic shuttling defect remains to be seen.

Several other lines of evidence suggest that components of the nucleo-cytoplasmic transport machinery play a role in efficient checkpoint function. In *Xenopus* extracts, the checkpoint is sensitive to Ran-GTP levels, with the addition of exogenous RCC1 resulting in the mis-localization of Bub1, Bub3, CENP-E and Mad2 and abrogation of checkpoint arrest in response to nocodazole (Arnaoutov & Dasso 2003). RanBP2/Nup358 also localizes to nuclear pores during interphase and to kinetochores during mitosis (Salina *et al.* 2003, Joseph *et al.* 2002). Interestingly, repression of RanBP2 inhibits chromosome congression and reduces kinetochore localization of Mad1, Mad2, CENP-F, CENP-E and Zw10. Several other observations connect the nuclear envelope with kinetochores and the checkpoint: Mad1 and Mad2 localize to nuclear pores during interphase (Campbell *et al.* 2001) and two other nucleoporins, hNup107 and hNup133, localize to kinetochores during mitosis (Belgareh *et al.* 2001). CENP-F is tightly bound to the nuclear matrix during interphase but then localizes to kinetochores in mitosis (Liao *et al.* 1995). However, just prior to mitotic entry CENP-F localizes to the nuclear envelope (Hussein & Taylor, 2002). Indeed, CENP-F is farnesylated, a modification that is often used to target proteins to membranes, and this modification appears to be required not only for CENP-F's ability to target the nuclear envelope but also for kinetochore localization in mitosis (Hussein & Taylor 2002). Farnesylation of CENP-F is also required for its degradation after mitosis. While the significance of these observations is unclear, it is intriguing that farnesylation of CENP-F is required for progression through G2. Coupled with the observation that Rae1 genetically interacts with the *Aspergillus* mitotic regulator NimA (Wu *et al.* 1998), these observations suggest that there may be some functional cross-talk between nuclear pores, kinetochores and mitotic entry.

The spindle checkpoint and cancer

The majority of human cancer cells are aneuploid due to an underlying chromosomal instability phenotype (Lengauer *et al.* 1997). Because inhibition of the spindle checkpoint yields a similar phenotype (Taylor & McKeon 1997, Michel *et al.*

2001), it is possible that CIN arises as a consequence of checkpoint defects *in vivo* (Cahill *et al.* 1998). However, mutations in checkpoint components are rare in human tumours. One possibility is that there are many genes required for chromosome stability and mutation in any one is sufficient to induce CIN. Alternatively, it is possible that inactivation of the checkpoint leads to such a high level of CIN that it does not offer a selective advantage. Consistently, homozygous mutation of Mad2, Bub3 and Rae1 leads to embryonic lethality in mice (Dobles *et al.* 2000, Kalitsis *et al.* 2000, Babu *et al.* 2003). Indeed, aneuploid colon cancer cells which exhibit CIN do arrest in mitosis when exposed to microtubule toxins (Tighe *et al.* 2001), suggesting that the checkpoint is not totally defective in these cells. Consequently, it is conceivable that either the genetic lesions that give rise to CIN are more subtle than inactivating mutations, or the mutations are in genes that are not involved in the spindle checkpoint *per se* but rather modulate the checkpoint in a subtle way. Significantly, Aurora A maps to chromosome 20q13, a region amplified in a variety of human cancers and consistently Aurora A is over expressed in many tumours (Bischoff *et al.* 1998). 3T3 cells overexpressing a wild-type Aurora A, but not a kinase mutant, readily form tumours in nude mice. Furthermore, ectopic overexpression of Aurora A in cultured cells leads to transformation, centrosome abnormalities and aneuploidy (Zhou *et al.* 1998). In addition, quantitative trait loci mapping has identified Aurora A as a low-penetrance tumour-susceptibility gene in both mice and humans (Ewart Toland *et al.* 2003). While most depletion experiments implicate Aurora A in centrosome function and mitotic entry, overexpression of Aurora A has been reported to compromise the spindle checkpoint (Anand *et al.* 2003). Aurora A also binds Cdc20 (Farruggio *et al.* 1999) although the significance of this is not clear.

Recent observations indicate that mutations in the adenomatous polyposis coli (APC) protein may result in chromosome instability, at least in colon cancer. While APC's tumour suppressor role in the Wnt signalling pathway is well established, it is becoming clear that APC plays several roles in regulating the cytoskeleton. APC localizes to microtubules and binds EB1, a protein implicated in spindle assembly positioning (Tirnauer & Bierer 2000). Mouse embryonic stem cells harbouring homozygous APC mutations frequently become tetraploid (Fodde *et al.* 2001, Kaplan *et al.* 2001). Interestingly, APC localizes to kinetochores in mitosis and is a Bub1 substrate, suggesting that APC mutations may directly affect chromosome segregation and/or the spindle checkpoint. More recently it has been shown that APC mutation weakens kinetochore–microtubule interactions (Green & Kaplan 2003) and spindle structure (Dikovskaya *et al.* 2004). While APC may not therefore play a direct role in the checkpoint, it is possible that by compromising kinetochore–microtubule interactions, APC mutation may give rise to a low-level checkpoint defect that does offer a selective advantage during tumour evolution.

Finally, the fact that the checkpoint is functional in the majority of tumour cells analysed (Tighe *et al.* 2001) opens up the possibility of targeting the checkpoint in order to develop novel anti-cancer drugs. Indeed, it has recently been shown that it is possible to selectively inhibit Aurora kinase activity in cells with a small molecule inhibitor (Ditchfield *et al.* 2003, Hauf *et al.* 2003). These inhibitors compromise the spindle checkpoint: exposed cells prematurely undergo anaphase and mitotic exit. Importantly, whereas non-dividing cells retain viability in the presence of Aurora inhibitors, cycling cells rapidly lose viability (Ditchfield *et al.* 2003). Furthermore, relative to cells with a functional p53 response, p53-deficient cells are more likely to continue cell cycle progression in the presence of an Aurora inhibitor. Significantly, Aurora inhibitors also appear to exhibit anti-tumour activity *in vivo* (Harrington *et al.* 2004). Therefore, regardless of whether or not Aurora A, B or C is directly required for spindle checkpoint function, these observations suggest that targeting the spindle checkpoint may open up new opportunities to develop novel anticancer agents.

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