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chapter 43

The mitotic checkpoint: a signaling pathway that allows a single unattached kinetochore to inhibit mitotic exit

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The mitotic checkpoint is a failsafe mechanism for the cell to ensure accurate chromosome segregation during mitosis. Mutations in genes encoding essential checkpoint proteins lead to chromosome instability and promote carcinogenesis. The BUB and MAD genes are essential components of the mitotic checkpoint pathway. BUB and MAD inhibit the ubiquitin ligase activity of the Anaphase Promoting Complex/Cyclosome (APC/C) during mitosis to ensure cells with unaligned chromosomes do not prematurely enter anaphase. Two models explain how the APC/C is inhibited by the checkpoint. The Sequestration Model postulates that Mad2 and BubR1 bind and sequester Cdc20, an APC/C activator, away from APC/C so substrates whose destruction drives mitotic exit are no longer ubiquitinated. In this model, the unattached kinetochore is postulated to catalytically convert Mad2 to a form that binds Cdc20. In the Direct Inhibition Model, the Mitotic Checkpoint Complex (MCC) consisting of BubR1, Bub3, Mad2 and Cdc20 binds and inhibits the APC/C independently of the kinetochore. However, the "wait anaphase" signal generated by unattached kinetochores sensitizes the APC/C to prolonged inhibition by the MCC. A single unattached kinetochore is proposed to amplify the "wait anaphase" signal through a kinase cascade involving checkpoint kinases such as hBubR1, hBub1 and Mps1.

Mitosis occupies the shortest period in the cell cycle, and for most metazoans, is completed within one to two hours. During this time, chromosomes must establish microtubule connections to the separated spindle poles so that they can align at the spindle equator. Attachment of chromosomes to the spindle relies on chance encounters between microtubules and a chromosomal structure called the kinetochore (1, 2). The stochastic nature of chromosome alignment poses a mitotic cell with the tremendously important and difficult problem of monitoring the actions of all the chromosomes to ensure that they are all properly aligned before they can separate and allow the cell to exit mitosis. This task is mediated by a checkpoint mechanism that directly monitors kinetochore microtubule attachments (3, 4). The cell keeps track of all kinetochores by assigning checkpoint proteins to each one so that its activity can be directly monitored. This mechanism can account for how even a single unaligned chromosome can block cells from exiting mitosis (5). In addition to playing a localized role in monitoring kinetochore activities, checkpoint proteins must also operate at a global level to block the degradation of proteins that inhibit entry into anaphase. In many ways, the mitotic checkpoint pathway resembles classic signal transduction cascades in that a signal that is generated at a restricted area of the cell must be amplified through effector molecules to alter the global biochemical status of the cell. Many molecules important for this checkpoint have been identified. The challenge is to understand how these proteins function at the various steps to connect unattached kinetochores with the Anaphase Promoting Complex/Cyclosome (APC/C) that promotes mitotic exit through ubiquitin-mediated proteolysis.

THE CHECKPOINT MONITORS KINETOCHORE TENSION AND MICROTUBULE OCCUPANCY

The checkpoint discriminates between aligned and unaligned chromosomes by relying on differences in kinetochore chemistry. Kinetochores of aligned chromosomes are saturated with microtubules and tension develops between the sister kinetochores as poleward directed forces try to pull them apart (6-8). By contrast, unaligned chromosomes have far fewer kinetochore microtubules and the kinetochore experiences virtually no tension. The idea that the checkpoint was sensitive to kinetochore tension was elegantly demonstrated in a series of micromanipulation experiments that was performed on the trivalent sex chromosomes in the mantis spermatocyte (9). During meiosis I, the two X chromosomes pair with a single Y chromosome. On occasion, one of the X-chromosomes fails to achieve bipolar microtubule attachment and its mono-orientation delays anaphase. If tension was applied to the unattached kinetochore with a microneedle, the block to anaphase was lifted and all the chromosomes that were at the spindle equator separated and moved poleward.

Despite these results, evidence also indicates that the checkpoint is sensitive to microtubule occupancy at kinetochores. Mad2 is a checkpoint protein that binds to unattached kinetochores but is released once kinetochores become aligned (10, 11). To test whether Mad2 binding to kinetochores is sensitive to tension or microtubule occupancy, cells were treated with the microtubule stabilizing drug, taxol. Because taxol suppresses the poleward flux of tubulin subunits in the microtubule, the bipolar attached kinetochores are not under tension (as measured by the distance between sister kinetochores) even though their kinetochores are

saturated with microtubules. Under these circumstances, Mad2 was released from kinetochores of the aligned chromosomes presumably because it was sensitive to microtubule attachments (12).

While Mad2 appears to respond to microtubule occupancy at kinetochores, it is clear that other components of the checkpoint remain sensitive to the loss of tension because taxol- treated cells arrest in mitosis. Indeed, the "relaxed" kinetochores were found to exhibit phosphorylations that were recognized by the 3F3/2 phospho-specific monoclonal antibody (12, 13). Although the precise identity of the 3F3/2 epitope is unknown, the antibody recognizes a small set of mitotic phosphoproteins, some of which must be associated with kinetochores. The retention of 3F3/2 phosphorylation at the "relaxed" kinetochores supports micromanipulation experiments that revealed that 3F3/2 phosphorylation at kinetochores was sensitive to tension (14). Thus, there may be kinases at kinetochores whose activities are regulated by tension. The tension-sensitive kinases remain to be identified but likely candidates include Bub1, BubR1 and Mps1, all of which are critical for the mitotic checkpoint and are associated with kinetochores. The human BubR1 kinase is of particular interest as it was found to physically associate with CENP-E (15), a kinesin-like protein that is required for proper kinetochore-microtubule attachments and for kinetochore tension (15, 16).

MITOTIC CHECKPOINT PROTEINS BIND KINETOCHORES AND ALSO INHIBIT THE APC/C

The molecular components of the mitotic checkpoint were first discovered in budding yeast. In the presence of spindle or kinetochore defects, the Mps1 kinase (17-19) and the Bub1/Bub3 kinase complex (20, 21) along with Mad1, Mad2, Mad3, establish a pathway to block cells in mitosis (22, 23). Bub2 does not appear to monitor chromosome alignment as it has recently been shown to function during anaphase by coordinating spindle elongation with cytokinesis (24, 25). Genetic studies in both fission and budding yeast (21, 22) showed that the target of the spindle checkpoint is the Anaphase Promoting Complex (APC/C), a megadalton multisubunit protein complex that ubiquitinates proteins whose destruction is required for sister chromatid separation and exit from mitosis (26-28). Inhibition of the APC/C is thought to be mediated by the checkpoint protein Mad2 through its interaction with Cdc20 (29, 30), a substrate specificity factor of the APC/C (30, 31). However, more recent evidence indicates the mechanism of inhibition may not be so simple (See details below).

Many of the checkpoint proteins discovered in yeast have been conserved throughout evolution. Bub1, Bub3, Mad1, Mad2, Mad3 and Mps1 orthologs have been identified in metazoans and shown to be essential for the checkpoint (10, 11, 15, 32-43). One of the most revealing features of these checkpoint proteins is that they are localized to kinetochores where they are postulated to monitor the activity of individual kinetochores during chromosome alignment. Indeed, the finding that unattached kinetochores exhibited a higher level of checkpoint proteins than attached kinetochores suggested that these proteins are sensitive to kinetochore-microtubule interactions. Once chromosomes have achieved metaphase alignment, Mad1 (35) and Mad2 (11) are no longer detectable at the kinetochores, while Bub1 and BubR1 levels are reduced by three to four-fold (12, 36, 44).

CHECKPOINT CONTROL IN METAZOANS IS MORE COMPLEX

The spindle checkpoint pathways in yeast and metazoans share a common framework that has been conserved throughout evolution (3). However, the checkpoint mechanism in metazoans appears to be more complex. This is perhaps not surprising given that a more elaborate checkpoint system might be required to accommodate the increased complexity in the structure and function of metazoan kinetochores. Indeed, mammalian cells express two Bub1-related kinases, Bub1 and BubR1 (15, 32, 34, 38) instead of a single Bub1 kinase in budding yeast. Studies of mouse and human Bub1 (32, 34), and human BubR1 (45) have shown that both kinases are essential for the checkpoint. This suggests that Bub1 and BubR1 act in concert or along parallel pathways to mediate spindle checkpoint functions. ZW10 (zeste white 10) and Rod (rough deal) are essential components of the checkpoint but are only conserved amongst metazoans. These genes may have evolved in response to the ability of dynein to bind to kinetochores in metazoans.

hBUBR1 checkpoint function may be to monitor the kinetochore motor CENP-E

hBUBR1 was independently identified by three separate approaches. hBUBR1 was found in a screen for genes that are mutated in colorectal carcinomas (32) and in a directed search for mammalian homologs of the yeast Mad3 checkpoint protein (38). Clues to hBubR1 function came when it was discovered in a yeast two hybrid screen for proteins that interacted with the kinetochore- binding domain of CENP-E (15). CENP-E is a kinesin-like microtubule motor whose function at kinetochores is to mediate microtubule attachments and specify chromosome alignment (46-48). The function of CENP-E is likely to be monitored by the checkpoint as human cells invariably arrest in mitosis when their kinetochores lack CENP-E (45, 46, 48). The yeast two hybrid results were strengthened by the fact that CENP-E and hBubR1 formed a complex in HeLa cells (15). Furthermore, immuno-EM studies showed that hBubR1 was concentrated at the outer kinetochore plate (36) where CENP-E is also localized (49, 50). Based on these findings, CENP-E and hBubR1 were postulated to be integral parts of a mechanosensor that links kinetochore motility with checkpoint control (15, 45). In support of this, hBubR1 was indeed found to be an essential component of the spindle checkpoint in HeLa cells (45). Furthermore, hBubR1 is essential for the arrest that is mediated when CENP-E function is disrupted in HeLa cells (45). These findings are consistent with the

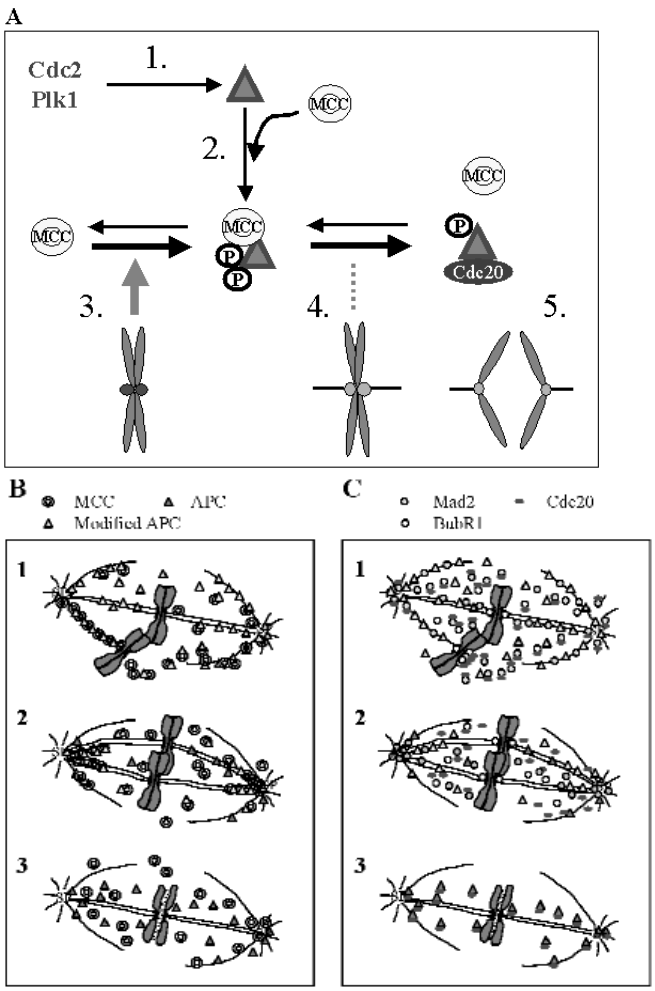

Figure 1.
Direct Inhibition Model of the BUB/MAD dependent checkpoint. (A) MCC is present thoughout the cell cycle. It is only active against APC/C that has undergone mitotic modifications. APC/C is localized at the spindle, spindle poles, kinetochores as well as the chromosomes during mitosis. APC/C is activated by phosphorylations and is competant to bind Cdc20 at the onset of mitosis (1). MCC recognizes these modifications and binds to the APC/C and blocks its from ubiquitinating its targets (2). The MCC:APC/C interaction is unstable so that inhibition of the APC is reversible. Signals initiated at unattached kinetochores can stabilize the interaction between MCC and APC/C (3). Microtubule attachments and/or kinetochore tension are probably monitored and transmitted through a kinetochore mechanosensory complex involving the BubR1 kinase and the kinetochore motor, CENP-E. We speculate that CENP-E regulates kinetochore tension and microtubule attachments that in turn affect the kinase activity of BubR1 that is associated at kinetochores. The wait anaphase signal is amplified through a kinase cascade involving both the kinetochore bound as well as the soluble pools of checkpoint kinases such as BubR1, Bub1 and Mps1. When all chromosomes are aligned at the metaphase plate, MCC and APC/C interactions are no longer stabilized by unattached kinetochores (4). APC/C is relieved from MCC inhibition and anaphase is allowed to proceed (5).
(B) 1. The presence of unattached kinetochore maintain MCC and APC/C interactions and APC/C activity is inhibited. The dynein/dynactin dependent transport of checkpoint proteins such as Mad2 and BubR1 (most likely as MCC) from kinetochores to spindle pole and the rapid kinetochore turnover rate might facilitate the amplification of the wait anaphase signal. **2.** When chromosomes are aligned, the signal that maintains MCC and APC/C interactions decays and MCC dissociates from the APC/C. 3. After APC/C is released from MCC. Sister chromatid separation can occur after Pds1 is degraded and Esp1 protease is free to cleave the cohesin Scc1/Mcd1/Rad21. SP is the spindle pole.
Sequestration Model of the BUB/MAD dependent checkpoint. (C) Unattached kinetochores are postulated to bind and convert Mad2 to a form that bind Cdc20 and sequesters it away from APC/C. In addition to Mad2, hBubR1 is also postulated to bind and sequester Cdc20 and thereby inhibit APC/C activity. **1.** In the presence of unattached kinetochores, Mad2 and hBUBR1binds to Cdc20 and prevents Cdc20from activating the APC/C. **2.** When all the chromosomes have aligned, Mad2 is transported off the kinetochores to the spindle pole in a dynein dependent manner. As active Mad2 are no longer being converted by unatttached kinetochores, Mad2 and hBubR1 dissociate from Cdc20. **3.** Cdc20 that is dissociated from its inhibitors can then recruit substrates to the APC/C.

idea that one function of BubR1 is to monitor CENP-E microtubule interactions at kinetochores. However, hBubR1 functions are complex and appear to play an additional role in inhibiting the APC/C (see below).

The finding that disruption of CENP-E functions in mammalian cells causes mitotic arrest conflicts with studies in *Xenopus* where depletion of CENP-E from egg extracts abrogated the spindle checkpoint (51). Checkpoint failure in the egg extracts is likely due to the fact that checkpoint proteins such as Mad2 cannot assemble onto kinetochores that lack CENP-E. The situation is drastically different in mammalian cells as all of the known checkpoint proteins were found to be present at kinetochores depleted of CENP-E in HeLa cells (48). We believe that the inactivation of the checkpoint in frog extracts is likely due to a general failure to assemble a kinetochore when CENP-E is absent. This possibility is supported by recent findings where depletion of Bub1 from egg extracts blocks not only assembly of checkpoint proteins onto kinetochores but also of CENP-E (52). This differs from HeLa cells where we found that hBub1 is not essential for CENP-E to bind to kinetochores even though it is critical for the assembly of Mad1, Mad2, hBubR1 and other checkpoint proteins to kinetochores (unpublished observations of Jablonski and Yen). Why kinetochore assembly is affected differently by CENP-E and Bub1 in frogs and mammals remain to be clarified but may reflect fundamental diffe-

rences in the organization of kinetochores between frogs and mammals. Alternatively, the kinetochore assembly pathways between embryonic and somatic cells may differ because of differences in cell cycle regulation. It is possible that to accommodate the rapid embryonic cell cycle, subcomplexes of the kinetochore are pre-assembled so that they can be rapidly recruited to the replicated centromeres. By contrast, somatic cells appear to assemble proteins to the kinetochore sequentially in a cell cycle-dependent manner (15, 36, 53).

hZW10 and hROD are novel components of the mitotic checkpoint

Drosophila ZW10 (zeste white 10) and ROD (rough deal) were identified as genes that were important for chromosome segregation (54, 55). ZW10 and ROD mutants exhibited identical phenotypes in which mutant embryos exhibited high rates of aneuploidy. Immunocytochemical studies showed that Zw10 and Rod are kinetochore proteins that recruit dynein to kinetochores (56). Interestingly, no apparent orthologs of Zw10 and Rod have been identified in budding yeast despite the fact that these genes are conserved in worms, vertebrates and plants (57, 58). We speculate that ZW10 and ROD appeared late in evolution at the time when dynein was recruited to kinetochores. Most cells in ZW10 and ROD null mutant flies achieve metaphase alignment in the absence of dynein at kinetochores and progress normally through mitosis. Nevertheless, there is an increase in defective anaphase cells with lagging chromosomes that is consistent with the cells prematurely exiting mitosis before chromosomes are properly aligned.

Indeed, recent studies of both human and *Drosophila* Zw10 and Rod indicate they are novel components of the checkpoint. Microinjection of hZw10 and hRod antibodies into HeLa cells blocked the assembly of hZw10, hRod and dynein/dynactin to kinetochores (59). The effects were very specific as these antibodies did not interfere with the localization of CENP-E, hBub1, hBubR1, Mad1 or Mad2 at kinetochores. Cells injected with hZw10 or hRod antibodies were found to divide with lagging chromosomes, consistent with premature exit from mitosis due to a defective checkpoint. To directly confirm that hZw10 and hRod are essential for the checkpoint, cells lacking hZw10/hRod at kinetochores were challenged with nocodazole and found to be unable to arrest in mitosis in the absence of a spindle. These cells exited mitosis without dividing, forming polyploid cells. The loss of checkpoint control was not due to the fact that disruption of hZw10 and hRod blocked the assembly of other checkpoint proteins to kinetochores. Indeed, we showed that not only were all of the known checkpoint proteins present at kinetochores, but they also appeared to remain sensitive to microtubule binding. In other words, checkpoint proteins such as Mad1, Mad2, hBubR1 and hBub1 remained sensitive to kinetochore-microtubule attachments (59). These proteins were found prominently at kinetochores that were not attached to the spindle, while kinetochores with bipolar attachments exhibited weak or undetectable levels of these proteins, as in normal metaphase kinetochores. This finding suggests that hZw10 and hRod are directly involved with the checkpoint rather than acting indirectly through other checkpoint proteins. A similar analysis of cells in zw10 and rod mutant flies showed an inability to arrest in mitosis when exposed to colchicine. Despite the absence of a spindle, these mutant cells degraded cyclin B1 and precociously separated the sister chromatids (60). This information demonstrated that the APC/C was activated in zw10 and rod mutants despite the presence of spindle defects.

MODELS FOR HOW THE SPINDLE CHECKPOINT INHIBITS THE ANAPHASE PROMOTING COMPLEX

Sequestration Model

Genetic studies in budding and fission yeast demonstrated that the target of the spindle checkpoint is the APC/C. Checkpoint defective alleles of cdc20/slp1 in budding and fission yeast, respectively showed that they failed to associate with Mad2. As Cdc20 is known to activate the APC/C by recruiting specific substrates to the APC/C, the data suggested that Mad2 inhibited the APC/C by sequestering its activator cdc20. *In vitro* studies of vertebrate Mad2 showed that it can block APC/C activity even when kinetochores are not present. Thus, addition of recombinant Mad2 will arrest frog egg extracts in a mitotic state even when chromosomes are omitted from the assay (35, 61). Likewise, recombinant Mad2 can directly inhibit APC/C ubiquitination activity *in vitro* (61, 62). In agreement with the yeast genetic data (30, 63), inhibition of the APC/C by recombinant Mad2 requires Cdc20 (61). One possibility is that Mad2 binds to Cdc20 and prevents it from presenting substrates to the APC/C.

The *in vitro* results along with *in vivo* studies of Mad2 in mammalian cells (10, 43) have led to a model whereby unattached kinetochores recruit Mad2 from the cytosol and convert it into a form that can inhibit the APC/C after its release from an unattached kinetochore (10, 43). Indeed, FRAP experiments showed that the turnover rate of Mad2 at unattached kinetochores is between 1000 to 2000 molecules per minute (64). While this data documents the turnover rate of Mad2 at kinetochores, the fate of the Mad2 that is released from kinetochores remains unknown. Furthermore, the nature of the modification that activates Mad2 is not known. A change in conformation of Mad2 whereby monomeric Mad2 is assembled into an active tetrameric form at unattached kinetochores has been proposed to activate Mad2 to inhibit APC/C (61, 65, 66). The hypothesis was largely based on the observation that bacterially expressed Mad2 readily forms tetramers (61). However, such Mad2 oligomers have not been observed in other systems (67, 68). The validity of the Mad2 oligomer hypothesis is now under question as it has been discovered that a Mad2 mutant that is unable to form oligomers can activate the mitotic checkpoint as well as the wild type Mad2 (69).

Recent *in vitro* studies have shown that recombinant hBubR1 can also bind Cdc20 and thus may act in conjunction with Mad2 to sequester Cdc20 away from APC/C. Indeed, recombinant hBubR1 was shown to inhibit the APC/C in a Cdc20-dependent fashion (70). Furthermore, recombinant hBubR1 and Mad2 were found to act synergistically to inhibit APC/C *in vitro* (71). It is important to note however, that these experiments used immunopurified interphase APC/C, whose activation depended on exogenously added Cdc20. Thus it is not surprising that addition of hBubR1 or Mad2 would titrate Cdc20 away from APC/C. It is not known whether sequestration of Cdc20 by Mad2 and hBUBR1 as shown in these studies reflects the mechanism by which the APC/C is inhibited inside the cell. This issue is of concern given that recombinant hBubR1 was found to be incapable of inhibiting its physiological target, the mitotic APC/C (70).

Direct Inhibition of the APC/C by the Mitotic Checkpoint Complex

Studies described above have led to the sequestration model whereby checkpoint proteins such as hBubR1 and Mad2 bind to Cdc20 and prevent it from recruiting substrates to the APC/C. However, the discovery of the Mitotic Checkpoint Complex (MCC) has lead to a different model whereby checkpoint proteins directly inhibit the APC/C. The MCC was biochemically isolated from HeLa cells as a factor that inhibited the ubiquitin ligase activity of mitotic APC/C (68). The inhibitory activity was found to co-fractionate with, and depend on, hBubR1. Furthermore, MCC consists of checkpoint proteins Bub3, Mad2 and Cdc20 in near equal stoichiometry. The MCC is evolutionarily conserved, a complex of Mad3, Bub3, Mad2 and Cdc20 having been identified in budding yeast (72) as well as fission yeast (73). However, conflicting reports have suggested that BubR1 does not form a complex with Mad2 in human cells (70, 71). Several lines of evidence have indicated the MCC in HeLa cells is likely the physiological inhibitor of the APC/C. Biochemical evidence showed that over half of the APC/C that was present in mitotic HeLa cells was physically associated with the MCC and this population exhibited low ubiquitin ligase activity as compared to the population of APC/C not associated with the MCC. MCC was found to be > 3000-fold more potent as an inhibitor of mitotic APC/C than recombinant Mad2. This is significant in light of the fact that the amount of Mad2 in HeLa cells that is not part of the MCC is no greater than 20 to 25-fold. Thus, the vast majority of the Mad2 in HeLa cells should not be able to inhibit the APC/C. Finally, the nearly equal stoichiometry of the MCC to APC/C indicates that there is sufficient concentration of the MCC in HeLa cells to bind and inhibit the cellular pool of APC/C (68).

Perhaps the most unexpected finding was that the MCC was present and fully active during interphase, when there are no functional kinetochores. Importantly, the MCC was found to only inhibit the mitotic form of the APC/C. The properties of MCC challenged the prevailing model that the inhibitor of the APC/C is generated from unattached kinetochores. The existence of a pre-formed pool of MCC is postulated to allow cells to rapidly inactivate APC/C once cells enter mitosis. By necessity, this inhibition must be highly reversible but can be prolonged in the presence of unattached kinetochores. This idea is consistent with the observation that the APC/C in lysates prepared from mitotically arrested HeLa cells was not permanently inhibited but regained activity after a 15 minute lag. The reactivation of the APC/C was not due to the decay of its inhibitor (*i.e.* MCC) as addition of purified chromosomes prolonged the inhibition for extended periods. These data suggested three possible ways by which chromosomes (kinetochores) enhanced the inhibition of the APC/C in the mitotic lysates. Firstly, chromosomes might inhibit the stimulatory activity of Cdc20 if it was indeed acting as a catalyst to convert proteins such as Mad2 into a form that sequesters Cdc20 away from the APC/C. Secondly, chromosomes might enhance the inhibitory activity of the MCC. Finally, the chromosomes might act on the APC/C in a way that sensitizes and prolongs its association with the MCC. These possibilities were tested by assessing the effects of incubating chromosomes with Cdc20, MCC, and the APC/C. The results showed that chromosomes neither suppressed the stimulatory activity of Cdc20 nor enhanced the inhibitory affects of the MCC. However, when mitotic APC/C (which contains MCC) was pre-incubated with chromosomes, the lag in its activity was significantly extended. This suggests that unattached kinetochores might biochemically modify the APC/C so that it is more sensitive to inhibition by the MCC. An important caveat of these reconstitution experiments is that kinetochores were assessed for their ability to modify MCC, Cdc20 or the APC/C directly. For example, the extended inhibition of the mitotically purified APC/C would require that the chromosomes act directly on the APC/C. It is unlikely *in vivo* that a single kinetochore could directly modify the global pool of APC/C. Thus, a key component missing in these reconstitution experiments is the amplification step that allows even a single chromosome to maintain the prolonged inhibition of the APC/C.

What is the nature of the amplification step? According to the Sequestration model, the unattached kinetochore acts in a catalytic fashion to convert the cellular pool of Mad2 into a form that sequesters Cdc20 and thus prevents activation of the APC/C. This is supported by the rapid turnover of Mad2 at unattached kinetochores. Implicit in this model is that the interaction between Cdc20 and Mad2 is inherently unstable so that checkpoint inhibition of the APC/C is reversible. It is interesting that recent studies have shown that Mad2 binds Cdc20 with a 10-fold higher affinity than Mad1. However, these measurements were made using recombinant Mad2 and peptide fragments of Cdc20 and Mad1 respectively. Full length Cdc20 does not bind Mad2 as well as the Mad2 binding domain in Cdc20 (74). Mad1 has been proposed to play a key role in the recruitment of Mad2 to kinetochores (35, 75) and is required for Mad2/cdc20 interaction *in vivo*

(30, 76). However, the mechanism by which Mad1 enables Mad2/Cdc20 interaction is unclear as Mad2/Cdc20 interaction occurs readily *in vitro* without Mad1. Careful measurements of the binding and dissociation rates between Mad2, full length Cdc20 and full length Mad1 should be highly illuminating.

The role of unattached kinetochores in the Direct Inhibition Model is proposed to sensitize the APC/C to prolonged inhibition by the MCC. The biochemical connection between kinetochores and the APC/C is envisioned to be a kinase cascade (68). Checkpoint kinases, such as Bub1, BubR1 and Mps1, are located at kinetochores and may be activated by loss of microtubule connections or reduction of kinetochore tension. In this regard, the kinetochore-associated hBubR1 kinase became quantitatively hyperphosphorylated within 15 min after disruption of microtubules (45). Assuming that mitosis-specific activity of the BubR1 kinase is mediated by phosphorylation, the activated BubR1 kinase might initiate the "wait anaphase" signal. The "wait anaphase" signal can be amplified through a kinase cascade involving the non-kinetochore-associated pool of BubR1, Bub1 or Mps1 checkpoint kinases, or other kinases. Phosphorylation of the APC/C by these kinases is then predicted to stabilize the interaction between MCC and the APC/C so that APC/C activity is inhibited. Regardless of the identity of the kinases that lie downstream of the kinetochore, their activities must be intrinsically short-lived so that once the "wait anaphase" is extinguished, the kinase cascade will also be rapidly extinguished. Likewise, the modifications (phosphorylations) that sensitize APC/C to prolonged inhibition by the MCC must also be inherently labile so that APC/C is not permanently inhibited by the MCC. The finding that the PP5 phosphatase is associated with APC/C subunits (77), Cdc27 and Cdc16, suggests that it may dephosphorylate the residues in APC/C subunits which when phosphorylated render it sensitive to MCC inhibition.

Combined biochemical, genetic and cell biological studies have contributed important molecular insights into the mitotic checkpoint pathway. These studies have also shown that this pathway is complex and highly regulated in order that a cell will delay mitotic exit even when there is a single unaligned chromosome. While there is general agreement that checkpoint proteins monitor kinetochore microtubule attachments and tension, views differ on how kinetochores inhibit the APC/C. The Sequestration Model posits that unattached kinetochores catalytically convert proteins such as Mad2 to bind Cdc20 and prevent it from recruiting substrates to the APC/C. On the other hand, the Direct Inhibitor Model posits that a pre-formed pool of MCC inhibitor directly binds and blocks APC/C activity. Unattached kinetochores generate a kinase cascade to modify the APC/C so that it is sensitized to prolonged inhibition by the APC/C. Which of these two mechanisms describe the situation *in vivo* remains to be sorted out but it is also possible that both pathways operate in a redundant way to ensure APC/C is not prematurely activated until all chromosomes are properly aligned.

MITOTIC CHECKPOINT PROTEINS AS CANCER DRUG TARGETS

Aneuploidy is a hallmark of a many cancer cells postulated to be due to mutations in the mitotic checkpoint control genes (32). In fact, somatic mutations of hBUB1 or hBUBR1 were found in 4/19 colorectal cancer cell lines that exhibited the chromosome instability (CIN) phenotype but mutations in other mitotic checkpoint genes (MAD1, MAD2, BUB3, MPS1, and CDC20) in these 19 cell lines were not detected (78). Since then, various groups have screened a variety of cancer cell lines for mutations in mitotic checkpoint genes but mutations have not been found (79-96), even though many of these cancer cells are defective in the mitotic checkpoint (83, 87). In tumors of homozygous mutant Brca2 mice, Bub1 is found to be mutated (97). Homozygous Mad2 knockout in mice results in embryonic lethality, indicating that a functional mitotic checkpoint is essential (98). Furthermore, human and mouse embryonic cell lines that are heterozygous for Mad2 display CIN and premature anaphase. Mice that are heterozygous for Mad2 have a high rate of tumor development after long latency, implicating defective mitotic checkpoints in tumor formation (99). In *C. elegans*, Mad2 is essential (100). In *Drosophila*, mutations in Bub1 cause chromosome missegregation and apoptosis in neuroblast cells (42). Although mutations appear to be rare in tumor cells displaying the CIN phenotype, mutations in mitotic checkpoint genes can cause chromosome instability. Since defective mitotic checkpoint and chromosome instability are hallmarks of cancer cells, the mitotic checkpoint genes provide prime targets for the development of drugs that specifically targets cancer cells. Jallepalli and Lengauer have proposed to use a cell-based screening assay to identify compounds that selectively kill cells with a mitotic checkpoint defect (101). Paired isogenic human cancer cell lines differing only in the presence or absence of a K-Ras mutant were used in drug screening (102). The screening assay uses BFP and YFP as color markers for the two cell lines. The cell lines were mixed equally in 96-well plates and the growth curve of each cell line determined by fluorometry. A novel cytidine nucleoside analog was identified that selectively inhibits growth of the cells containing the mutated K-Ras allele versus the K-Ras knockout cells. Similar drug screens using cell lines with engineered mutation in the mitotic checkpoint pathway should also be possible. Initial screening could also be carried out in yeast as the mitotic checkpoint pathway is well conserved and cell based drug screens using yeast mutants have been performed successfully (103, 104). By combining yeast and vertebrate cell-based screens, compounds have been identified that selectively kill cells with DNA repair deficiency (105). It would be of interest to develop a screen using cell lines that contain Mad1, Bub1 or BubR1 mutations.

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REFERENCES

1. Nicklas, R. B. (1997) *Science* **275**, 632-637.
2. Yen, T. J., and Schaar, B. T. (1996) *Curr Opin Cell* Biol **8**, 381-388.
3. Skibbens, R. V., and Hieter, P. (1998) A*nnu Rev Genet* **32**, 307-337.
4. Rieder, C. L., Schultz, A., Cole, R., and Sluder, G. (1994) *J Cell Biol* **127**, 1301-1310.
5. Rieder, C. L., Cole, R. W., Khodjakov, A., and Sluder, G. (1995) *J Cell Biol* **130**, 941-948.
6. Cassimeris, L., Rieder, C. L., Rupp, G., and Salmon, E. D. (1990) *J Cell Sci* **96**, 9-15.
7. McEwen, B. F., Heagle, A. B., Cassels, G. O., Buttle, K. F., and Rieder, C. L. (1997) *J Cell Biol* **137**, 1567-1580.
8. Zhai, Y., Kronebusch, P. J., and Borisy, G. G. (1995) *J Cell Biol* **131**, 721-734.
9. Li, X., and Nicklas, R. B. (1995) *Nature* **373**, 630-632.
10. Li, Y., and Benezra, R. (1996) *Science* **274**, 246-248.
11. Chen, R. H., Waters, J. C., Salmon, E. D., and Murray, A. W. (1996) *Science* **274**, 242-246.
12. Waters, J. C., Chen, R. H., Murray, A. W., and Salmon, E. D. (1998) *J Cell Biol* **141**, 1181-1191.
13. Campbell, M. S., and Gorbsky, G. J. (1995) *J Cell Biol* **129**, 1195-1204.
14. Nicklas, R. B., Ward, S. C., and Gorbsky, G. J. (1995) *J Cell Biol* **130**, 929-939.
15. Chan, G. K., Schaar, B. T., and Yen, T. J. (1998) *J Cell Biol* **143**, 49-63.
16. McEwen, B. F., Chan, G. K., Zubrowski, B., Savoian, M. S., Sauer, M. T., and Yen, T. J. (2001) *Mol Biol Cell* **12**, 2776-2789.
17. Winey, M., Goetsch, L., Baum, P., and Byers, B. (1991) *J Cell Biol* **114**, 745-754.
18. Lauze, E., Stoelcker, B., Luca, F. C., Weiss, E., Schutz, A. R., and Winey, M. (1995) *Embo J* **14**, 1655-1663.
19. Weiss, E., and Winey, M. (1996) *J Cell Biol* **132**, 111-123.
20. Hoyt, M. A., Totis, L., and Roberts, B. T. (1991) *Cell* **66**, 507-517.
21. Roberts, B. T., Farr, K. A., and Hoyt, M. A. (1994) *Mol Cell Biol* **14**, 8282-8291.
22. Li, R., and Murray, A. W. (1991) *Cell* **66**, 519-31.
23. Hardwick, K. G., and Murray, A. W. (1995) *J Cell Biol* **131**, 709-720.
24. Gardner, R. D., and Burke, D. J. (2000) *Trends Cell Biol* **10**, 154-158.
25. Burke, D. J. (2000) *Curr Opin Genet Dev* **10**, 26-31.
26. King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. (1996) *Science* **274**, 1652-9.
27. Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V., and Hershko, A. (1995) *Mol Biol Cell* **6**, 185-97.
28. King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M. W. (1995) *Cell* **81**, 279-88.
29. Kallio, M., Weinstein, J., Daum, J. R., Burke, D. J., and Gorbsky, G. J. (1998) *J Cell Biol* **141**, 1393-1406.
30. Hwang, L. H., Lau, L. F., Smith, D. L., Mistrot, C. A., Hardwick, K. G., Hwang, E. S., Amon, A., and Murray, A. W. (1998) *Science* **279**, 1041-1044.
31. Visintin, R., Prinz, S., and Amon, A. (1997) *Science* **278**, 460-463.
32. Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Willson, J. K., Markowitz, S. D., Kinzler, K. W., and Vogelstein, B. (1998) *Nature* **392**, 300-303.
33. Basu, J., Logarinho, E., Herrmann, S., Bousbaa, H., Li, Z., Chan, G. K., Yen, T. J., Sunkel, C. E., and Goldberg, M. L. (1998) *Chromosoma* **107**, 376-385.
34. Taylor, S. S., and McKeon, F. (1997) *Cell* **89**, 727-735.
35. Chen, R. H., Shevchenko, A., Mann, M., and Murray, A. W. (1998) *J Cell Biol* **143**, 283-295.
36. Jablonski, S. A., Chan, G. K., Cooke, C. A., Earnshaw, W. C., and Yen, T. J. (1998) *Chromosoma* **107**, 386-396.
37. Jin, D. Y., Spencer, F., and Jeang, K. T. (1998) *Cell* **93**, 81-91.
38. Taylor, S. S., Ha, E., and McKeon, F. (1998) *J Cell Biol* **142**, 1-11.
39. Campbell, M. S., Chan, G. K., and Yen, T. J. (2001) *J Cell Sci* **114**, 953-63.
40. Fisk, H. A., and Winey, M. (2001) Cell **106**, 95-104.
41. Abrieu, A., Magnaghi-Jaulin, L., Kahana, J. A., Peter, M., Castro, A., Vigneron, S., Lorca, T., Cleveland, D. W., and Labbe, J. C. (2001) *Cell* **106**, 83-93.
42. Basu, J., Bousbaa, H., Logarinho, E., Li, Z., Williams, B. C., Lopes, C., Sunkel, C. E., and Goldberg, M. L. (1999) *J Cell Biol* **146**, 13-28.
43. Gorbsky, G. J., Chen, R. H., and Murray, A. W. (1998) *J Cell Biol* **141**, 1193-1205.
44. Hoffman, D. B., Pearson, C. G., Yen, T. J., Howell, B. J., and Salmon, E. D. (2001) *Mol Biol Cell* **12**, 1995-2009.
45. Chan, G. K., Jablonski, S. A., Sudakin, V., Hittle, J. C., and Yen, T. J. (1999) *J Cell Biol* **146**, 941-954.
46. Schaar, B. T., Chan, G. K., Maddox, P., Salmon, E. D., and Yen, T. J. (1997) *J Cell Biol* **139**, 1373-8132.
47. Wood, K. W., Sakowicz, R., Goldstein, L. S., and Cleveland, D. W. (1997) *Cell* **91**, 357-366.
48. Yao, X., Abrieu, A., Zheng, Y., Sullivan, K. F., and Cleveland, D. W. (2000) *Nat Cell Biol* **2**, 484-491.
49. Yao, X., Anderson, K. L., and Cleveland, D. W. (1997) *J Cell Biol* **139**, 435-447.
50. Cooke, C. A., Schaar, B., Yen, T. J., and Earnshaw, W. C. (1997) *Chromosoma* **106**, 446-455.
51. Abrieu, A., Kahana, J. A., Wood, K. W., and

Cleveland, D. W. (2000) *Cell* **102**, 817-826.

52. Sharp-Baker, H., and Chen, R. H. (2001) *J Cell Biol* **153**, 1239-1250.
53. Liao, H., Winkfein, R. J., Mack, G., Rattner, J. B., and Yen, T. J. (1995) *J Cell Biol* **130**, 507-518.
54. Karess, R. E., and Glover, D. M. (1989) *J Cell Biol* **109**, 2951-2961.
55. Williams, B. C., Karr, T. L., Montgomery, J. M., and Goldberg, M. L. (1992) *J Cell Biol* **118**, 759-773.
56. Starr, D. A., Williams, B. C., Hays, T. S., and Goldberg, M. L. (1998) *J Cell Biol* **142**, 763-774.
57. Scaerou, F., Aguilera, I., Saunders, R., Kane, N., Blottiere, L., and Karess, R. (1999) *J Cell Sci* **112**, 3757-3768.
58. Starr, D. A., Williams, B. C., Li, Z., Etemad-Moghadam, B., Dawe, R. K., and Goldberg, M. L. (1997) *J Cell Biol* **138**, 1289-301.
59. Chan, G. K., Jablonski, S. A., Starr, D. A., Goldberg, M. L., and Yen, T. J. (2000) *Nat Cell Biol* **2**, 944-947.
60. Basto, R., Gomes, R., and Karess, R. E. (2000) *Nat Cell Biol* **2**, 939-943.
61. Fang, G., Yu, H., and Kirschner, M. W. (1998) *Genes Dev* **12**, 1871-1883.
62. Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M., and Benezra, R. (1997) *Proc Natl Acad Sci USA* **94**, 12431-12436.
63. Kim, S. H., Lin, D. P., Matsumoto, S., Kitazono, A., and Matsumoto, T. (1998) *Science* **279**, 1045-1047.
64. Howell, B. J., Hoffman, D. B., Fang, G., Murray, A. W., and Salmon, E. D. (2000) *J Cell Biol* **150**, 1233-1250.
65. Fang, G., Yu, H., and Kirschner, M. W. (1999) *Philos Trans R Soc Lond B Biol Sci* **354**, 1583-1590.
66. Fang, G., Yu, H., and Kirschner, M. W. (1998) *Mol Cell* **2**, 163-171.
67. Luo, X., Fang, G., Coldiron, M., Lin, Y., Yu, H., Kirschner, M. W., and Wagner, G. (2000) *Nat Struct Biol* **7**, 224-229.
68. Sudakin, V., Chan, G. K., and Yen, T. J. (2001) *J Cell Biol* **154**, 925-936.
69. Sironi, L., Melixetian, M., Faretta, M., Prosperini, E., Helin, K., and Musacchio, A. (2001) Embo J **20**, 6371-6382.
70. Tang, Z., Bharadwaj, R., Li, B., and Yu, H. (2001) *Dev Cell* **1**, 227-237.
71. Fang, G. (2002) *Mol Biol Cell* **13**, 755-766.
72. Hardwick, K. G., Johnston, R. C., Smith, D. L., and Murray, A. W. (2000) *J Cell Biol* **148**, 871-882.
73. Millband, D. N., and Hardwick, K. G. (2002) *Mol Cell Biol* **22**, 2728-27242.
74. Zhang, Y., and Lees, E. (2001) *Mol Cell Biol* **21**, 5190-5199.
75. Chung, E., and Chen, R. H. (2002) *Mol Biol Cell* **13**, 1501-1511.
76. Fraschini, R., Beretta, A., Sironi, L., Musacchio, A., Lucchini, G., and Piatti, S. (2001) *Embo J* **20**, 6648-6659.
77. Ollendorff, V., and Donoghue, D. J. (1997) *J Biol Chem* **272**, 32011-32018.
78. Cahill, D. P., da Costa, L. T., Carson-Walter, E. B., Kinzler, K. W., Vogelstein, B., and Lengauer, C. (1999) *Genomics* **58**, 181-187.
79. Shichiri, M., Yoshinaga, K., Hisatomi, H., Sugihara, K., and Hirata, Y. (2002) *Cancer Res* **62**, 13-17.
80. Shigeishi, H., Oue, N., Kuniyasu, H., Wakikawa, A., Yokozaki, H., Ishikawa, T., and Yasui, W. (2001) *Pathobiology* **69**, 24-29.
81. Shigeishi, H., Yokozaki, H., Kuniyasu, H., Nakagawa, H., Ishikawa, T., Tahara, E., and Yasui, W. (2001) *Oncol Rep* **8**, 791-794.
82. Reis, R. M., Nakamura, M., Masuoka, J., Watanabe, T., Colella, S., Yonekawa, Y., Kleihues, P., and Ohgaki, H. (2001) *Acta Neuropathol* (Berl) **101**, 297-304.
83. Ouyang, B., Knauf, J. A., Ain, K., Nacev, B., and Fagin, J. A. (2002) *Clin Endocrinol* (Oxf) **56**, 341-350.
84. Ohshima, K., Haraoka, S., Yoshioka, S., Hamasaki, M., Fujiki, T., Suzumiya, J., Kawasaki, C., Kanda, M., and Kikuchi, M. (2000) *Cancer Lett* **158**, 141-150.
85. Nomoto, S., Haruki, N., Takahashi, T., Masuda, A., Koshikawa, T., Fujii, Y., and Osada, H. (1999) *Oncogene* **18**, 7180-7183.
86. Olesen, S. H., Thykjaer, T., and Orntoft, T. F. (2001) *Carcinogenesis* **22**, 813-815.
87. Saeki, A., Tamura, S., Ito, N., Kiso, S., Matsuda, Y., Yabuuchi, I., Kawata, S., and Matsuzawa, Y. (2002) *Cancer* **94**, 2047-2054.
88. Sato, M., Sekido, Y., Horio, Y., Takahashi, M., Saito, H., Minna, J. D., Shimokata, K., and Hasegawa, Y. (2000) *Jpn J Cancer Res* **91**, 504-509.
89. Yamaguchi, K., Okami, K., Hibi, K., Wehage, S. L., Jen, J., and Sidransky, D. (1999) *Cancer Lett* **139**, 183-187.
90. Mimori, K., Inoue, H., Alder, H., Ueo, H., Tanaka, Y., and Mori, M. (2001) *Oncol Rep* **8**, 39-42.
91. Lin, S. F., Lin, P. M., Yang, M. C., Liu, T. C., Chang, J. G., Sue, Y. C., and Chen, T. P. (2002) *Leuk Lymphoma* **43**, 385-391.
92. Jaffrey, R. G., Pritchard, S. C., Clark, C., Murray, G. I., Cassidy, J., Kerr, K. M., Nicolson, M. C., and McLeod, H. L. (2000) *Cancer Res* **60**, 4349-4352.
93. Gemma, A., Hosoya, Y., Seike, M., Uematsu, K., Kurimoto, F., Hibino, S., Yoshimura, A., Shibuya, M., Kudoh, S., and Emi, M. (2001) *Lung Cancer* **32**, 289-295.
94. Gemma, A., Seike, M., Seike, Y., Uematsu, K., Hibino, S., Kurimoto, F., Yoshimura, A., Shibuya, M., Harris, C. C., and Kudoh, S. (2000) *Genes Chromosomes Cancer* **29**, 213-218.
95. Hernando, E., Orlow, I., Liberal, V., Nohales, G., Benezra, R., and Cordon-Cardo, C. (2001) *Int J Cancer* **95**, 223-227.
96. Haruki, N., Saito, H., Harano, T., Nomoto, S., Takahashi, T., Osada, H., and Fujii, Y. (2001)

Cancer Lett **162**, 201-205.

97. Lee, H., Trainer, A. H., Friedman, L. S., Thistlethwaite, F. C., Evans, M. J., Ponder, B. A., and Venkitaraman, A. R. (1999) *Mol Cell* **4**, 1-10.
98. Dobles, M., Liberal, V., Scott, M. L., Benezra, R., and Sorger, P. K. (2000) *Cell* **101**, 635-645.
99. Michel, L. S., Liberal, V., Chatterjee, A., Kirchwegger, R., Pasche, B., Gerald, W., Dobles, M., Sorger, P. K., Murty, V. V., and Benezra, R. (2001) *Nature* **409**, 355-359.
100. Kitagawa, R., and Rose, A. M. (1999) *Nat Cell Biol* **1**, 514-521.
101. Jallepalli, P. V., and Lengauer, C. (2001) *Nature* Rev Cancer **1**, 109-117.
102. Torrance, C. J., Agrawal, V., Vogelstein, B., and Kinzler, K. W. (2001) *Nat Biotechnol* **19**, 940-945.
103. Oliver, S. G. (1997) *Curr Opin Genet Dev* **7**, 405-409.
104. Ma, D. (2001) *Prog Drug Res* **57**, 117-162.
105. Dunstan, H. M., Ludlow, C., Goehle, S., Cronk, M., Szankasi, P., Evans, D. R., Simon, J. A., and Lamb, J. R. (2002) *J Natl Cancer Inst* **94**, 88-94.