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Identification of the Kinetochore Generated “Wait-Anaphase” Signal
of the Mitotic Checkpoint

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Biological Sciences

by

Anita Kulukian

Committee in charge:

Professor Don Cleveland, chair
Professor Steve Dowdy
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Professor Vivek Malhotra

2008

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Chair

University of California, San Diego

2008

Dedication

This work is dedicated to Annie Maben, Christine Hodson-Burt, Kevork Agopian, Anita Joukjian, Hagop Hagopian, and Leo Connolly, great teachers and educators who guided me, inspired me, excited me about science, and encouraged me to reach as high as I possibly could. Vartzgernit gadar.

This work is also dedicated to my family, for all their endless support.

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The text of Chapter 4 will be submitted for publication once the research has been completed. Joo Seok Han and I contributed equally to the research for this project, while I was the primary author. Don Cleveland directed and supervised the research that forms the basis of Chapter 4.

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Abstract of Dissertation

Identifying the Kinetochore Generated “Wait-Anaphase” Signal of the Mitotic Checkpoint

by

Anita Kulukian

Doctor of Philosophy in Biological Sciences

University of California, San Diego, 2008

Professor Don Cleveland, Chair

To ensure accurate segregation, the major cell cycle control mechanism in mitosis, the mitotic checkpoint, delays anaphase onset until all chromosomes have properly attached to spindle microtubules. This is achieved through the production of a “wait anaphase” inhibitor(s) that blocks recognition of cyclin B and securin by Cdc20-activated APC/C, an E3 ubiquitin ligase which targets them for destruction. Using physiologically relevant levels of Mad2, Bub3, BubR1, and Cdc20, unattached kinetochores on purified chromosomes are demonstrated to catalyze generation of a soluble Cdc20 inhibitor or inhibition of Cdc20 already bound to APC/C. Antibody inhibition of Mad1 and dimerization deficient Mad2 are used to demonstrate that the chromosome-produced inhibitor requires both recruitment of Mad2 by Mad1 stably bound at unattached kinetochores and dimerization competent Mad2. By acting directly on Mad2, but not BubR1, purified chromosomes promote

BubR1 binding to Cdc20 and APC/C, supporting a model in which immobilized Mad1/Mad2 at kinetochores provides a template for initial assembly of Mad2 bound to Cdc20 that is then converted to BubR1-Cdc20 as sequentially produced mitotic checkpoint inhibitors.

CHAPTER 1.

Introduction to the Mitotic Checkpoint

Mitosis: A chromosome segregation event monitored by the spindle checkpoint.

Cellular division is required for the propagation of almost every organism and forms the basis of multicellular life. Mitosis is the process by which a growing cell divides into two new daughter cells, each containing the same genetic information as the parent cell. While it comprises only a fraction of a cell's actual life cycle, it is one of its most crucial steps.

Mitosis is a beautifully coordinated event, with distinct progressive phases defining each stage. During prophase, DNA condenses into compacted chromosomes, with identical duplicated sisters joined together at the centromere and held together by cohesion. The centrosomes migrate away from each other to opposite ends of the cell and start nucleating microtubule fibers which begin to form the mitotic spindle. As the cell moves into prometaphase, the spindle becomes fully bipolar and the nuclear envelope is broken down. Condensed chromosomes form attachments to the spindle. Spindle microtubules emanating from the centrosomes attach to specialized structures called kinetochores found on each sister chromatid. Chromosome pairs form bipolar spindle attachments, with each chromatid

attached to the opposite spindle pole. Metaphase defines the moment when all chromosomes have completed this task, with tension aligning them at the metaphase plate of the cell. Cohesion amongst the duplicated chromatids is then dissolved, and the two sisters are pulled to opposite poles in anaphase. The nuclear envelope reforms around the segregated chromosomes which decondense in telophase. The cleavage furrow ingresses to complete cytokinesis, or the partitioning of the cytoplasm, to produce two new cells.

Before progressing from metaphase to anaphase, a surveillance mechanism exists to ensure accurate chromosome segregation and thus maintain the integrity of the genome in progeny cells. This mechanism is known by a variety of names, including the mitotic checkpoint or the spindle assembly checkpoint. Without a functional checkpoint, errors in chromosome segregation may occur which can lead to cell death (Kops et al., 2004). Cells which remain viable after cytokinesis often do so with an aberrant number of chromosomes (Weaver et al., 2003; Weaver et al., 2007). Non-disjunction of a particular chromosome results in a number of well characterized defects, including Down and Klinefelter Syndrome. Chromosome missegregation can lead to dosage differences in tumor suppressor genes or oncogenes, contributing to genomic instability (Yuen et al., 2005). Aneuploidy, or the condition of having an abnormal number of chromosomes, has long been noted as a hallmark of cancer.

To prevent the potential consequences of chromosome missegregation errors, the mitotic checkpoint halts the progression from metaphase to anaphase until all chromosomes form proper bipolar attachment to the spindle (Figure 1). The checkpoint monitors the spindle attachment status of chromosomes. This is accomplished in a two-fold manner. It senses occupancy of microtubules at the kinetochores. It also senses tension that is generated when chromosomes are bipolarly attached to spindle microtubules, which depolymerize at the pole and pull along the chromosomes in the process (Pinsky and Biggins, 2005). The checkpoint is activated in response to chromosomes which are unattached to the spindle (Rieder et al., 1995; Rieder et al., 1994). Incorrect attachments such as syntellic ones in which both kinetochores of sister chromatids are bound to the same spindle pole do not generate tension, but also activate the checkpoint. Conversely, merotelic attachments, with one kinetochore bound to fibers leading to both poles, create sufficient tension to escape checkpoint regulation and result in mitotic segregation errors (Cimini et al., 2001).

Kinetochores as sensors/generators of the mitotic checkpoint.

The kinetochore is the proteinaceous structure which attaches to spindle microtubules and directs chromosome movement during mitosis. It assembles at the site of constriction of a duplicated chromosome that is formed by heterochromatin (Cleveland et al., 2003). This region is called the

centromere, and while its exact location is determined by more than a DNA sequence in higher organisms, it is generally specified by the replacement of histone H3 by the histone H3 variant CenpA in nucleosomes around which DNA is packaged (Dalal et al., 2007; Heit et al., 2006; Vos et al., 2006). The heterochromatin forms the inner centromeric platform onto which over 80 proteins assemble in an interdependent and hierarchical manner in eukaryotes. Composed of structural elements, kinases, microtubule motors, and checkpoint controlling proteins, kinetochores form an ultrastructure of an inner and outer kinetochore region flanked by a fibrous corona observable only on microtubule unattached kinetochores (Cheeseman and Desai, 2008; Dong et al., 2007).

Aside from making connection to the spindle microtubules, kinetochores have been suggested to be the site of production of the mitotic checkpoint. Unattached kinetochores were first implicated as essential for generation of the anaphase inhibitor by observing that anaphase onset proceeded microtubule capture of the last unattached kinetochore within a specified amount of time lasting approximately twenty minutes (Rieder et al., 1994). Laser ablation of the last unattached kinetochore allows anaphase to ensue within the same span of time (Rieder et al., 1995) and so does the application of force produced by a microneedle pressing on a kinetochore to mimic tension experienced when kinetochores form microtubule attachments (Li and Nicklas, 1995). Manipulation of the last remaining unattached kinetochore to

break or prevent its spindle microtubule connection delays anaphase indefinitely (Li and Nicklas, 1995). Spindle checkpoint function in budding yeast requires the formation of a functional kinetochore (Gardner et al., 2001; Pangilinan and Spencer, 1996). Phosphorylation of 3F3/2 epitope marking unattached kinetochores (Gorbsky JCB 1993) is coincident with checkpoint activation or lack of tension and is lost after checkpoint satisfaction (Li and Nicklas, 1997). Additionally, a number of proteins have been identified which accumulate at unattached kinetochores either constitutively or dynamically (Howell et al., 2000; Howell et al., 2004; Kallio et al., 2002; Shah et al., 2004) and contribute to the production of checkpoint arrest, providing a molecular link between kinetochores and checkpoint activation.

Checkpoint proteins: Kinetochore-localized orchestrators of the wait anaphase signal.

Two screens for components which arrest a cell in mitosis in response to spindle damage in *Saccharomyces cerevisiae* led to the identification of MAD1,2,3 (Mitotic Arrest Deficient) (Hoyt et al., 1991) and BUB1,2,3 (Budding Uninhibited by Benamidazole) (Li and Murray, 1991) genes. Since then, a number of additional proteins have been identified which regulate mitotic progression by activating the spindle checkpoint. Homologs of these proteins, with the exception of Bub2, were found in a number of higher organisms, demonstrating evolutionary conservation of the mitotic checkpoint pathway

(Musacchio and Salmon, 2007). Bub2, which does not have a eukaryotic homolog, has since been demonstrated to localize to spindle poles and functions in a pathway separate from the mitotic checkpoint (Fraschini et al., 1999) to monitor spindle positioning in anaphase (Bloecher et al., 2000). RNAi depletion of these proteins accelerates progression through metaphase, with insufficient time allowed for forming microtubule attachments by chromosomes, especially in response to spindle damage (Meraldi et al., 2004). Consequently, chromosomes segregation errors are characteristic their impeded function. In mouse models, homozygous deletion of these genes always results in embryonic lethality, while hypomorph or heterozygous animals have increased rates of aneuploidy and tumor incidence (Baker et al., 2005). Lesions in these genes have been found in a variety of human cancers, including breast, lung, and intestinal cancer (Wang et al., 2008).

Mad-s and Bub-s.

Mad1 and Mad2 form a tight complex (Sironi et al., 2001) and relocalize from the nuclear envelope to unattached kinetochores in prometaphase (Campbell et al., 2001; Chen et al., 1996). Mad1 is a large approximately 90kD coiled-coil hyperphosphorylated protein (Hardwick and Murray, 1995) that recruits Mad2 to the kinetochores (Chen RH JCB 1999). Mad1 itself requires Bub1 for its localization (Sharp-Baker and Chen, 2001). Antibody injection against either Mad1 in *Xenopus* extracts (Chen et al., 1999) or Mad2 in Ptk1 and keratinocytes (Gorbsky et al., 1998) induced premature anaphase onset,

with chromosomes which had not aligned at the metaphase plate. Both Mad1 and Mad2 deletions in mice embryos lead to embryonic lethality (Dobles et al., 2000; Iwanaga et al., 2007), and are haploinsufficient (Iwanaga et al., 2007; Michel et al., 2001). Mad2 overexpression also leads to high rates of aneuploidy and tumorigenesis (Sotillo et al., 2007).

Mad3 has homologs in the fission yeast *S.Pombe*, and in the nematode *C.elegans*, and with the evolution of its kinase domain, is known as BubR1 in higher eukaryotes (Taylor et al., 1998). Its homolog Bub1 is also a kinase (Roberts et al., 1994) that is required for the checkpoint (Basu et al., 1999; Chen, 2004; Taylor and McKeon, 1997). Like BubR1/Mad3, it too localizes to kinetochores (Taylor and McKeon, 1997). Bub1 functions in part to protect centromeric cohesion among sister chromatids by aiding the localization of Shugoshin (Kitajima et al., 2004; Tang et al., 2004b). Disruption of Bub1 by P-element insertion in *Drosophila* (Basu et al., 1999), antisense in human cells (Musio et al., 2003), or by tamoxifen administration in mice (Perera et al., 2007) leads to chromosome missegregation and instability, with null alleles in mice leading to embryonic lethality. Bub1 hypomorph mice are viable and fertile, but have a high percentage of aneuploid cells and are more susceptible to developing tumors. Surprisingly, Bub1 heterozygous mice do not have a higher incidence of tumor development (Jeganathan et al., 2007). In contrast, BubR1 heterozygous MEFs develop lung tumors and intestinal carcinomas in response to carcinogens (Dai et al., 2004).

Both Bub1 and BubR1 localize to kinetochores by binding to and forming mutually exclusive complexes with Bub3 (Brady and Hardwick, 2000; Campbell et al., 2001; Fraschini et al., 2001; Martinez-Exposito et al., 1999; Roberts et al., 1994; Taylor et al., 1998). Bub3 is required for the checkpoint in budding yeast (Hoyt et al., 1991), flies (Basu et al., 1998), frogs (Campbell and Hardwick, 2003), mice (Martinez-Exposito et al., 1999), and humans (Taylor et al., 1998), though by one account is reportedly dispensable for checkpoint function in fission yeast (Tange and Niwa, 2008). Bub3 null mouse embryos accumulate mitotic errors including lagging chromosomes and the formation of chromosome bridges (Kalitsis et al., 2000), and are embryonic lethal (Babu et al., 2003; Kalitsis et al., 2000). Heterozygosity in mice leads to checkpoint defects, chromosome segregation errors, and greater susceptibility to DMBA induced tumorigenesis, and is thus haploinsufficient (Babu et al., 2003).

In addition to roles in chromosome congression and maintenance of sister chromatid cohesion, Mad-s and Bub-s assert checkpoint activation by inhibiting the ability of Cdc20 to activate the APC/C, which is discussed in greater detail below.

Motors

Microtubule attachment is crucial for the fidelity of chromosome segregation during mitosis. This function is mediated in part by the minus end directed motor CenpE (Thrower et al., 1995; Wood et al., 1997). CenpE is a flexible (Kim et al., 2008) kinetochore-associated kinesin-like protein present

only during mitosis (Yen et al., 1991; Yen et al., 1992). CenpE, localized to the fibrous corona of the kinetochore (Cooke et al., 1997; Yao et al., 1997), binds to depolymerizing microtubules (Liao et al., 1994; Lombillo et al., 1995), where it powers chromosome alignment to the metaphase plate and later aids in poleward movement during anaphase (Brown et al., 1996; Kapoor et al., 2006; Wood et al., 1997). Depletion of CenpE (by suppression of synthesis or deletion of the gene in mice) leads to unstable kinetochore-microtubule attachments and results in mono-oriented and missegregated chromosomes (Putkey et al., 2002; Weaver et al., 2003; Yao et al., 2000; Yao et al., 1997). It is essential for the checkpoint (Abrieu et al., 2000) by providing a link to the microtubule attachment state (Yao et al., 2000). It directly binds to BubR1 (Abrieu et al., 2000; Yao et al., 2000), and stimulates its checkpoint necessary kinase activity (Mao et al., 2003; Weaver et al., 2003). Microtubule capture by CenpE subsequently silences BubR1 kinase activity (Mao et al., 2005).

Kinases

Phosphorylation of components involved in mitosis dictates their activity and several key kinases serve that function to activate checkpoint signaling. In addition to Bub1 and BubR1, Mps1/Mph1 (Monopolar Spindle 1) is also such a kinase (Lauze et al., 1995). Though first identified for its role in spindle pole duplication in *S.cerivisiae* (Winey et al., 1991), it is now unclear whether Mps1 is required for centrosome duplication in mammalian systems (Fisk et al., 2003; Stucke et al., 2002). It is, however, essential for the checkpoint (Abrieu

et al., 2001; Hardwick et al., 1996; Weiss and Winey, 1996). It localizes to kinetochores upon phosphorylation of its S844 site by MAPK (Zhao and Chen, 2006), where enrichment mediates its trans auto-phosphorylation to become fully activated (Jelluma et al., 2008a; Kang et al., 2007). How it participates in the checkpoint is still unresolved, but it seems to play several roles. It interacts with (Jones et al., 1999) and phosphorylates the Dam1 complex which mediates kinetochore microtubule attachment in yeast to strengthen that binding interaction (Shimogawa et al., 2006). It also phosphorylates Mad1 (Hardwick et al., 1996), and is required for Mad1, Mad2 and CenpE recruitment to the kinetochores (Abrieu et al., 2001; Liu et al., 2003; Tighe et al., 2008). More recently, a role for Mps1 has been characterized for reorienting kinetochores with microtubule attachment errors (Maure et al., 2007). By phosphorylating Borealin/Dasra, a component of the Chromosomal passenger Complex (Ruchaud et al., 2007), it activates Aurora B kinase to correct those attachments (Jelluma et al., 2008b).

In recent years, additional kinases have been characterized for their role in the checkpoint. This includes TAO1, (Thousand And One) required for chromosome congression and checkpoint induced anaphase delay. It interacts with BubR1 and is required for the enrichment of Mad2 at kinetochores (Draviam et al., 2007). The Nek2 family, first characterized for its role in centrosome assembly (Faragher and Fry, 2003; Fry et al., 2000; Graf, 2002; Twomey et al., 2004), was demonstrated to have a role in premature

chromosome segregation (Lou et al., 2004; Sonn et al., 2004). It localizes to kinetochores, interacts with Mad1, and is required for Mad2 localization to kinetochores (Lou et al., 2004). When depleted from cells, it impairs checkpoint response to spindle damage induced by the microtubule destabilizing drug nocodazole. Further work showed that it phosphorylates Sgo1 (Fu et al., 2007), suggesting that it regulates Sgo1 mediated sister-chromatid cohesion at centromeres. It also phosphorylates Hec1 to potentially regulate kinetochore-microtubule attachment stability (Du et al., 2008). Additionally, PRP4 (pre-messenger RNA processing 4) and Chk1, involved in the response to DNA damage, have been suggested to play a role in the maintenance of the mitotic checkpoint (Montembault et al., 2007; Zachos et al., 2007).

Others

Zw10, Zwilch, and Rod are also required for the checkpoint (Basto et al., 2000; Chan et al., 2000; Wang et al., 2004). They are necessary for accurate chromosome segregation (Williams et al., 1992), tension sensing (Williams et al., 1996), and are involved in the regulation of force responsible for poleward chromosome movement (Savoian et al., 2000) through their recruitment of the microtubule motor Dynein and its associated Dynactin to kinetochores (Starr et al., 1998). First identified in *Drosophila* (Williams et al., 1992), they have no obvious yeast homologs. They interact directly and form the RZZ complex (Kops et al., 2005; Scaerou et al., 1999; Scaerou et al.,

2001; Williams et al., 2003). They are recruited to unattached kinetochores via interaction with Hec1/Ndc80 and Zwint (Famulski et al., 2008; Kops et al., 2005; Lin et al., 2006) in response to an Aurora dependent tension sensing mechanism (Famulski and Chan, 2007). The RZZ is also required for the localization of Mad1-Mad2 complex to unattached kinetochores (Buffin et al., 2005; Kops et al., 2005).

PICH, a helicase member of SNF2 ATPase family, is a substrate of Plk, is also required for the spindle checkpoint. It is required for the KT localization of Mad2 and is proposed to bind to catenated DNA to monitor tension between two sister kinetochores (Baumann et al., 2007).

APC/C^{Cdc20}: Mitotic progressor.

Progression from metaphase to anaphase requires the deactivation of the master kinase cyclin-dependent kinase Cdk1 and the dissolution of cohesion among sister chromatids (Figure 2). Cdk1 activity is stimulated by binding to M-phase cyclins, and it is their degradation that drives the cell cycle. Degradation of cyclin B in particular is required for the metaphase to anaphase transition. On the other hand, removal of chromosome cohesion occurs through the cleavage of the Scc1 subunit of cohesin rings, which link sister chromatids together (Campbell and Cohen-Fix, 2002; Losada, 2007). This is accomplished by the proteolytic activity of Separase/Pds2/Cut1. Separase is kept inactive by its regulatory subunit Securin/Pds1/Cut2. Securin degradation

activates Separase, and allows for the separation of sister chromatids (Uhlmann, 2003). It is then the degradation of both Cyclin B and Securin that mediates the transition into anaphase (Murray, 2004; Pines, 2006; Yu, 2007) .

Proteasome mediated degradation of both cyclin B and securin (Cohen-Fix et al., 1996; Funabiki et al., 1996; Hershko and Ciechanover, 1998; Hershko et al., 1994), as well as additional substrates promoting mitotic progression, is accomplished by the multisubunit Anaphase Promoting Complex / Cyclosome (APC/C) (Glotzer et al., 1991; Hagting et al., 2002). Its cell cycle regulated ubiquitin ligase activity was first identified in 1995 by the fractionation of clam oocyte extracts, and a number of its components were identified for their role in stabilizing cyclins (yeast *cdc16*, *cdc23*, and *cse1* - (Irniger et al., 1995); *cdc27*, *cdc16* in *Xenopus* - (King et al., 1995); human *cdc27*, *cdc16* (Tugendreich et al., 1995)). At least thirteen subunits of the APC/C have been identified to date (*Xenopus* APC1-8 (Peters et al., 1996); human Cdc23 (Yu et al., 1998); Doc1/APC10 (Grossberger et al., 1999); APC11 (Gmachl et al., 2000); and *cdc26*, APC13 (Yoon et al., 2002)). Subunits APC2 and APC11 interact directly to form the catalytic core of the APC/C (Tang et al., 2001b). APC2 and APC 11 have homology with the cullin family of ubiquitin ligases (Yu et al., 1998), and to the Zn²⁺ binding RING-H2 finger proteins (Gmachl et al., 2000), respectively, making them members of the cullin-RING family of E3 ligases. Doc1 aids in the processivity of substrates (Carroll and Morgan, 2002). The remaining subunits play structural

roles via the scaffolding created by their TPR motifs (Passmore et al., 2005), upon which APC/C co-factors can bind (Vodermaier et al., 2003). All together, APC/C has a mass of 1.5-1.7kD (Dube et al., 2005; Passmore et al., 2005; Sudakin et al., 1995) and forms an asymmetric triangular structure with a hollow tube in the center, as revealed by cryo-EM (Passmore et al., 2005). As an E3 ligase of the ubiquitin-transfer cascade, APC/C works in conjunction with a non-specific E1 and the UBC family of E2 proteins (E2-C in clam (Hershko et al., 1994), UBC4, UbcX in *Xenopus* (Yu et al., 1996), Vihar in *Drosophila* (Mathe et al., 2004), and UbcH10 in humans (Bastians et al., 1999; Townsley et al., 1997).

APC/C is present throughout the cell cycle, but requires a co-factor to stimulate its ubiquitin ligase activity. In mitosis, this is accomplished by Cdc20/Slp1/p55/*fizzy* (Dawson et al., 1995; Lim et al., 1998; Lorca et al., 1998; Visintin et al., 1997; Weinstein, 1997), which is then replaced by Cdh1/HCT/*fizzy-related* (Schwab et al., 1997; Sigrist and Lehner, 1997; Zachariae et al., 1998a) as cells exit mitosis and enter the G1 phase (Irniger and Nasmyth, 1997). Along with the yeast meiosis-specific Ama (Cooper et al., 2000), this family of APC/C activators encodes for seven WD40 repeats at their C-termini which bind to APC/C directly (Fang et al., 1998b; Zachariae et al., 1998b). These co-factors mediate substrate recognition for the APC/C, with Cdc20 recognizing only substrates containing Destruction Box motifs (consensus sequence of RxxLxxxN (Fang et al., 1998b; Glotzer et al., 1991;

Sudakin et al., 1995)), while Cdh1 also recognizes KEN box containing (consensus sequence KENxxxN/D) substrates (Fang et al., 1998b; Pflieger and Kirschner, 2000).

Human APC/C is predominantly localized to centrosomes and the mitotic spindle (Tugendreich et al., 1995; Kraft et al., 2003). However, several of its subunits (APC1,3,6) are also enriched on unattached kinetochores, in an Aurora kinase dependent manner, leading to the question of whether it is sensitized there for inhibition by components of the checkpoint or by its phosphorylation state (Acquaviva et al., 2004). APC/C activity coincides with its phosphorylation in mitosis (Kramer et al., 1998; Kramer et al., 2000; Lahav-Baratz et al., 1995; Peters et al., 1996). APC/C subunits are phosphorylated on at least 34 mitosis specific sites, 15 of which are phosphorylated by Cdk1 *in vitro* and 3 by Plk1 (Kraft et al., 2003). However, it is unclear to what degree APC/C activity is dependent upon its phosphorylation. It is reported that its activity is enhanced by it (Kramer et al., 1998; Kramer et al., 2000; Rudner and Murray, 2000). The affinity of Cdc20 to the APC/C is increased with phosphorylation (Kramer et al., 2000; Rudner and Murray, 2000; Shteinberg et al., 1999), with the Cdk1 sites being sufficient for its binding (Kraft et al., 2003). Additionally, affinity for D-Box motifs is higher for mitotic, phosphorylated APC/C (Yamano et al., 2004). However, the core of APC/C activity is stimulated by the binding of Cdc20 (Fang et al., 1998b), and thus is differentially regulated by the availability and regulation of Cdc20.

Cdc20: Target of the mitotic checkpoint.

Rather than directly inhibiting the APC/C, it is Cdc20 that is the target of the checkpoint (Hwang et al., 1998). The checkpoint regulates the ability of Cdc20 to activate the APC/C, and checkpoint proteins function by curtailing Cdc20 activity.

Cdc20 is phosphorylated in mitosis (Kramer et al., 2000), in part by MAPK in *Xenopus* extracts (Chung and Chen, 2003), by Cdc2 *in vitro* (Kramer et al., 2000), and also by Bub1 (Tang et al., 2004a). It is speculated to be a substrate of BubR1 as well (Wu et al., 2000). While Cdc20 phosphorylation is neither required nor necessary for APC/C activation (Kramer et al., 2000), its phosphorylation makes it more susceptible to checkpoint regulation (Chung and Chen, 2003; Tang et al., 2004a). Bub1 kinase activity towards Cdc20 is stimulated during checkpoint activation and Bub1 phosphorylated Cdc20 is less capable of activating APC/C *in vitro* (Tang et al., 2004a).

Cdc20 inhibition results from the direct binding of the checkpoint proteins. Mad2 binds directly to Cdc20 in response to checkpoint activation (Fang et al., 1998a; Kallio et al., 1998; Kim et al., 1998; Wassmann and Benezra, 1998) to inhibit APC/C mediated degradation of cyclin B (He et al., 1997; Li et al., 1997). Disruption of Mad2-Cdc20 interaction abrogates the checkpoint and leads to the stabilization of cyclin B (Kim et al., 1998).

Mad2 can undergo a large conformational change in which it alternates between two distinct conformations: the “open” (or “N1”) conformation that shifts to a “closed” (or “N2”) conformation upon binding to either Mad1 or Cdc20 by enclosing the seatbelt domain found at its carboxy terminal (Luo et al., 2000; Luo et al., 2002; Luo et al., 2004; Mapelli et al., 2007; Sironi et al., 2002). It is the closed conformation that is thought to be inhibitory by direct capture of Cdc20, thereby inhibiting its activation of APC/C (Luo et al., 2004).

The checkpoint is dependent upon Mad2 localization to unattached kinetochores by forming a tight complex with phosphorylated Mad1 (Chen et al., 1999; Chen et al., 1996; Li et al., 1997; Sironi et al., 2001). Yet, a second pool of kinetochore-unbound, free Mad2 in the open conformation can be found in the cytoplasm of checkpoint arrested cells and *Xenopus* extracts (Chung and Chen, 2002; Luo et al., 2004). Removal of the free pool of Mad2 abolishes checkpoint mediated arrest (Chung and Chen, 2002), leading to the theory that Mad2 localization to kinetochores through its interaction with Mad1 facilitates the formation of Mad2-Cdc20 complexes, by catalyzing its conformational change from the open to closed structures (Luo et al., 2002; Luo et al., 2004; Sironi et al., 2002). Mad2 localization to kinetochores is replenished by free Mad2. This theory, known as the Two-State model (Figure 3A), has one flaw: Mad2, which binds Cdc20 with the same seat-belt arm with which it binds Mad1 (Sironi et al., 2001), must dissociate from Mad1 in order to do so. Yet, the Mad1 and Mad2 interaction is a relatively stable association

($K_B = 0.96 \cdot 10^6 \text{ M}^{-1}$; $K_D = 1.04 \cdot 10^{-6} \text{ M}$) compared to Mad2 binding to Cdc20 ($K_B = 9.4 \cdot 10^6 \text{ M}^{-1}$; $K_D = 0.1 \cdot 10^{-6} \text{ M}$) (Sironi et al., 2002).

A solution to this problem came from the analysis of Mad2 dimerization. Seminal FRAP experiments demonstrated that two equally sized pools of kinetochore bound Mad2 exist, which are either stably associated or cycle on and off rapidly (Shah et al., 2004). FRAP of purified components has demonstrated recruitment of a rapidly exchangeable Mad2 by a second stably associated Mad2 (bound in the closed conformation to a fragment of Mad1) (Vink et al., 2006). Fractionation and coprecipitation of purified Mad2 mutants which were dimerization deficient or locked in the open conformation revealed that Mad2 bound to Mad1 in structurally heterogeneous dimers (De Antoni et al., 2005; Mapelli et al., 2006; Nezi et al., 2006). Additionally, dimerization deficient Mad2 mutants were unable to support checkpoint signaling in vivo (De Antoni et al., 2005; Nezi et al., 2006). These data have led to the proposal of a Mad2 template model (Figure 3B) for activation of Mad2 (De Antoni et al., 2005; Mapelli et al., 2007; Mapelli and Musacchio, 2007), which postulates that Mad1 recruits a stably-bound molecule of Mad2, which then itself recruits an open-conformer Mad2 from the cytosol to poise it for Cdc20 capture. This model also provides an explanation for why dimers rather than monomers of Mad2 are more potent inhibitors of Cdc20 (Fang et al., 1998a).

Yet, Mad2 itself cannot be the only stoichiometric inhibitor of Cdc20. Mad2 and Cdc20 are present at almost equivalent concentrations in a cell

(120nM and 100nM, respectively (Tang et al., 2001a), but only a fraction of Mad2 within a cell is active (Luo et al., 2004), with estimates of 20-40nM (Sudakin et al., 2001). BubR1 has also been demonstrated to bind to Cdc20 (Fang, 2002; Tang et al., 2001a; Wu et al., 2000) and by so doing, inhibits APC/C ubiquitination of substrates (Fang, 2002; Tang et al., 2001a) with twelve-fold more potency than Mad2 (Fang, 2002).

BubR1 is phosphorylated in mitosis (Chan et al., 1999; Hardwick et al., 2000) even in the absence of microtubule poisons (Taylor et al., 2001). Its phosphorylation is mediated by Mad1 (Chen, 2002) and partially attributable to Plk1/Plx1 (Elowe et al., 2007; Wong and Fang, 2007), both of which are also localized to kinetochores, and thus its localization to kinetochores directly mediates its phosphorylation there. However, it is unclear whether the phosphostatus of BubR1 regulates its activity in mitosis (Figure 4A).

While it is unknown what role BubR1 phosphorylation may play in its mitotic regulation, BubR1 kinase activity as a requirement for the checkpoint is contentious (Figure 4B). It is only active in mitosis, and is stimulated in response to spindle disruption (Chan et al., 1999). BubR1 kinase activity is stimulated by CenpE in *Xenopus* extracts and human cells (Mao et al., 2003; Weaver et al., 2003), and turns off upon microtubule capture by the motor (Mao et al., 2005). However, replacement of wild type BubR1 with either a kinase dead or kinase domain truncated mutant in *Xenopus* extracts restores the kinetochore localization of checkpoint proteins and the interactions

between Mad2, Bub3, and Cdc20 (Chen, 2002). Additionally, similar mutants can still bind to Cdc20 and inhibit APC/C activation *in vitro*, albeit in a kinetochore independent assay (Tang et al., 2001a).

Regardless of its phosphorylation state or its kinase activity, BubR1 has been shown to function synergistically with Mad2 *in vitro*, mutually promoting binding to Cdc20 (Fang, 2002). A complex of BubR1/Mad3 also containing Bub3, Cdc20 and Mad2, dubbed the Mitotic Checkpoint Complex (MCC), has been purified from both human cells (Sudakin et al., 2001) and yeast (Fraschini et al., 2001; Hardwick et al., 2000), and is reportedly 3000 fold more potent as an APC/C^{Cdc20} inhibitor than Mad2 alone (Sudakin et al., 2001). However, its production seems to be independent of checkpoint signaling. MCC can be found in interphase cells (Sudakin et al., 2001) and in the absence of a functional kinetochore (Fraschini et al., 2001).

Despite extensive research on checkpoint proteins which contribute to Cdc20 inhibition, many questions remain about the mitotic regulation of APC/C^{Cdc20} and the role that kinetochores play in the generation of an inhibitor during checkpoint activation that can restrict the ubiquitination activity of this mitotic progressor. The exact identity of the anaphase inhibitor and mechanism by which it inhibits APC/C^{Cdc20} are still unresolved.

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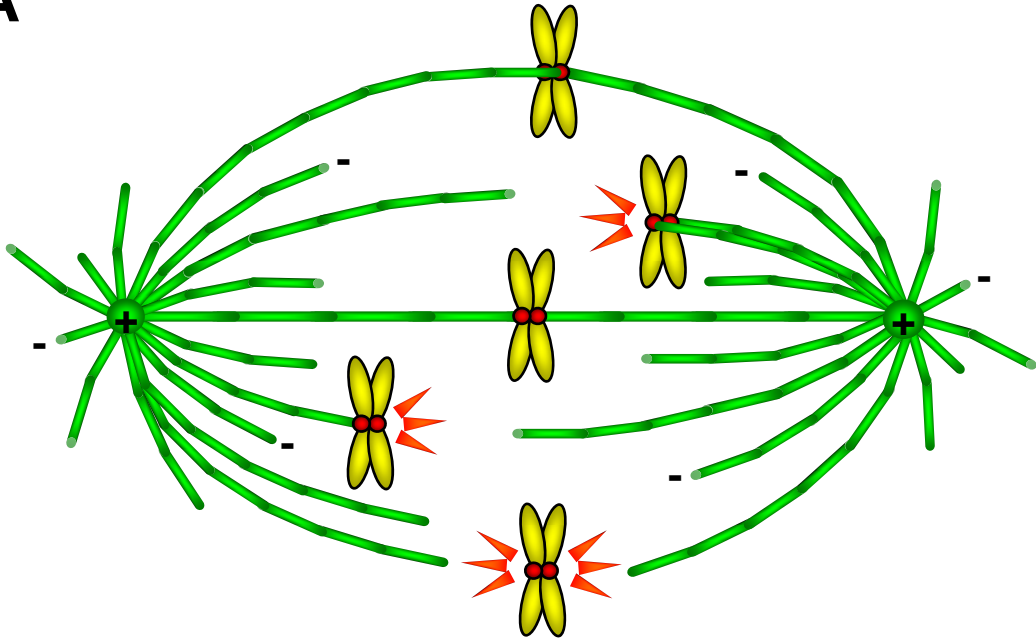
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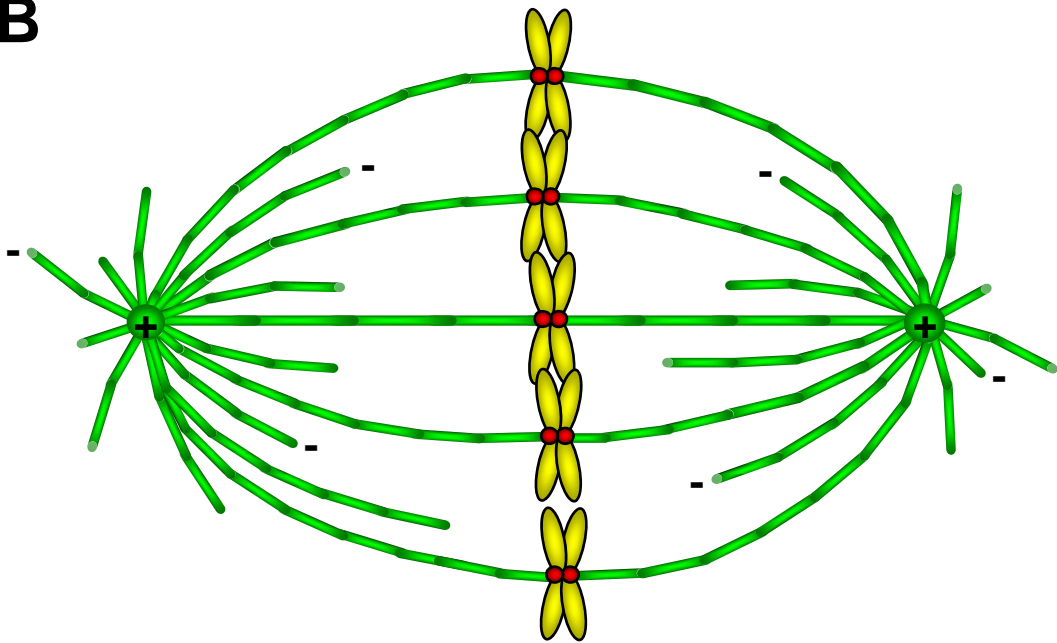
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Figure 1. The mitotic checkpoint is a surveillance mechanism which monitors chromosome attachment to the mitotic spindle.

(A) The mitotic checkpoint is active in the presence of chromosomes which have not formed tension producing bipolar attachment of their kinetochores, depicted in red, to microtubules emanating from the spindle, depicted in green. Kinetochores with syntellic attachments to the spindle, in which both kinetochores are attached to the same spindle, also activate the checkpoint, while kinetochores with merotellic attachments, in which one kinetochore is bound to microtubules emanating from both poles, produce adequate tension to bypass checkpoint activation. **(B)** The mitotic checkpoint is turned off when each and every kinetochore has formed bipolar attachment to the mitotic spindle.

A

Checkpoint "ON" State

B

Checkpoint "OFF" State

Figure 2. Transition from metaphase to anaphase is regulated by the degradation of cyclin B and securin.

(A) During prometaphase (when the mitotic checkpoint is active), the APC/C complex is held inactive such that it cannot ubiquitinated substrates such as cyclin B or securin. Cdk1 is active and cohesin rings keep sister chromatids bound to each other. **(B)** Once chromosomes have aligned at the metaphase plate and the checkpoint is satisfied, APC/C inhibition is relieved. Cyclin B and Securin are ubiquitinated and degraded, inactivating Cdk1 and activating Seperase. This in turn permits the cleavage of cohesin rings, allowing for the separation of sister chromatids, and pushes the cell into anaphase.

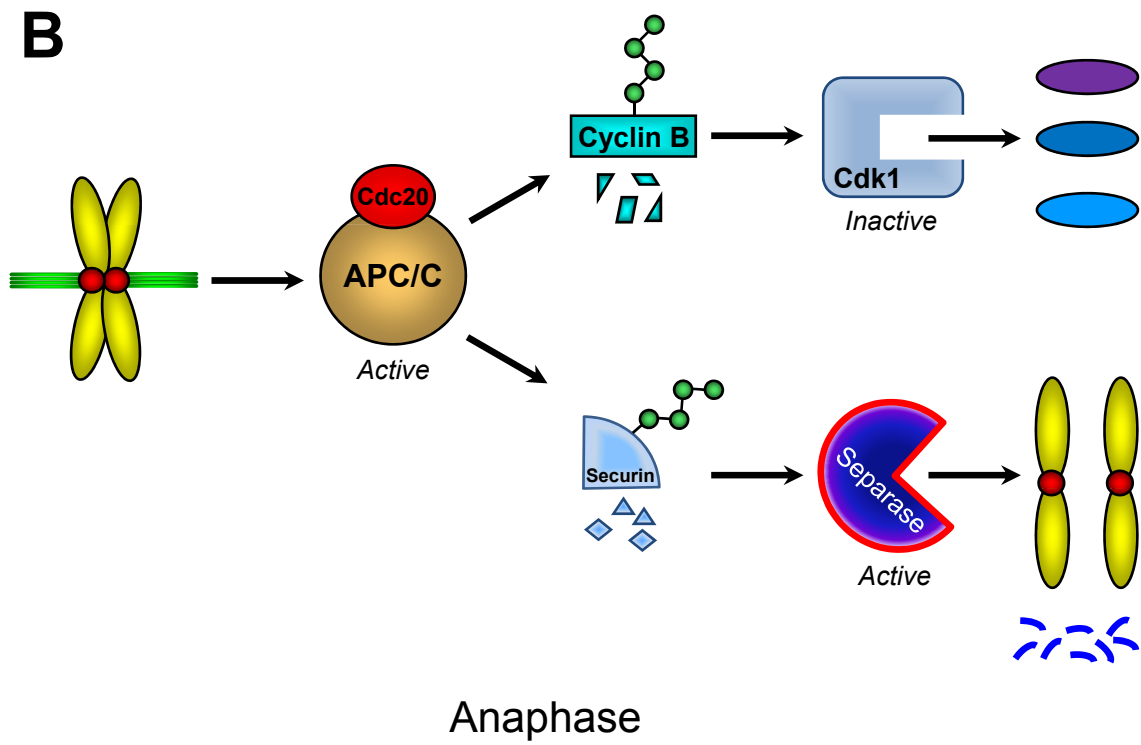
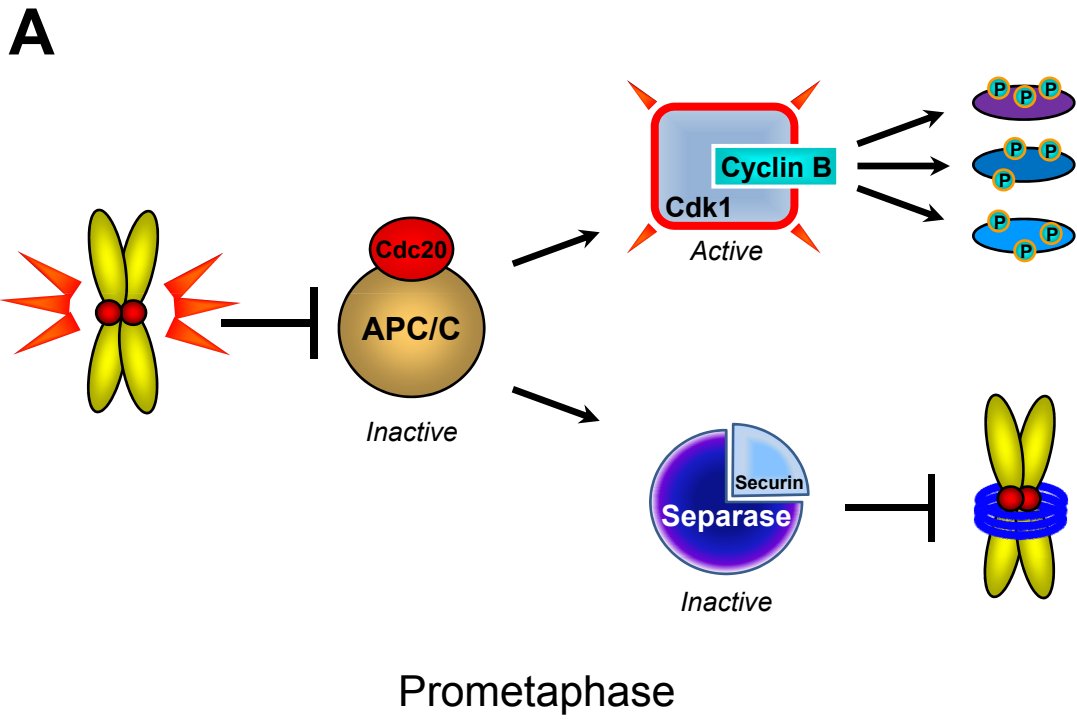
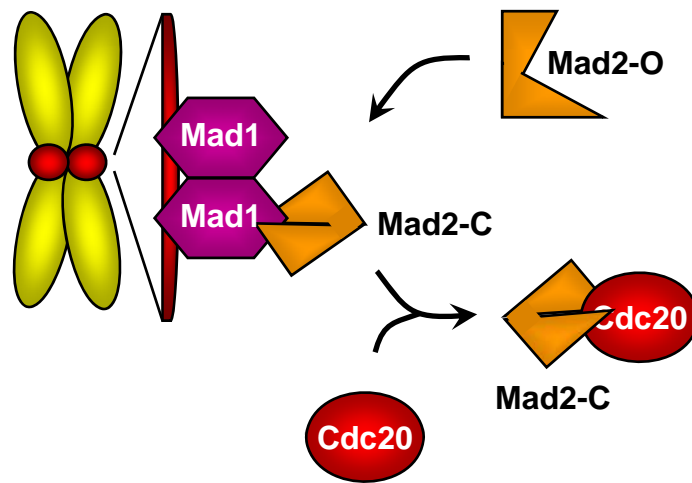


Figure 3. Mad2 models of activation.

(A) The original “Two-State” model predicted that Mad2 binding of Mad1 at kinetochores mediates its conformational change from an open structure to a closed one. Release of closed Mad2 from Mad1 promotes the binding of Mad2 to Cdc20 in the same binding pocket with which it bound to Mad1. **(B)** In contrast, the Template model proposes that Mad2 bound to Mad1 recruits open cytosolic Mad2. Dimerization of Mad2 promotes its conformational change to a conformer which can more readily bind to Cdc20.

A

The “Two-State” Model

**B**

The “Template” Model

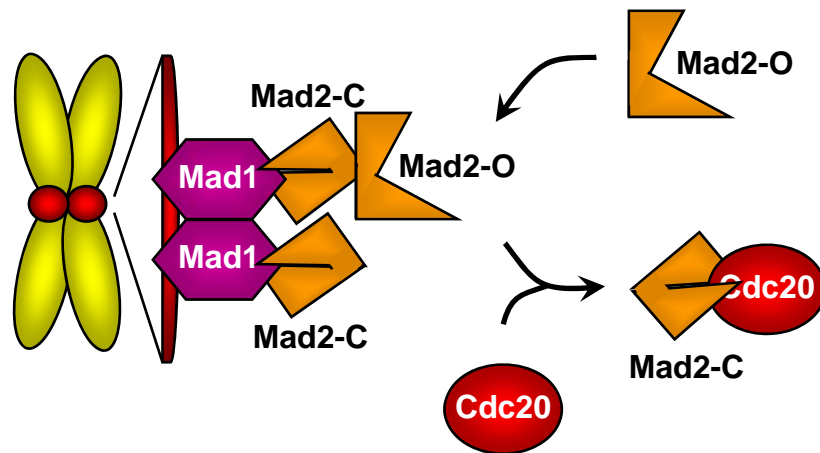
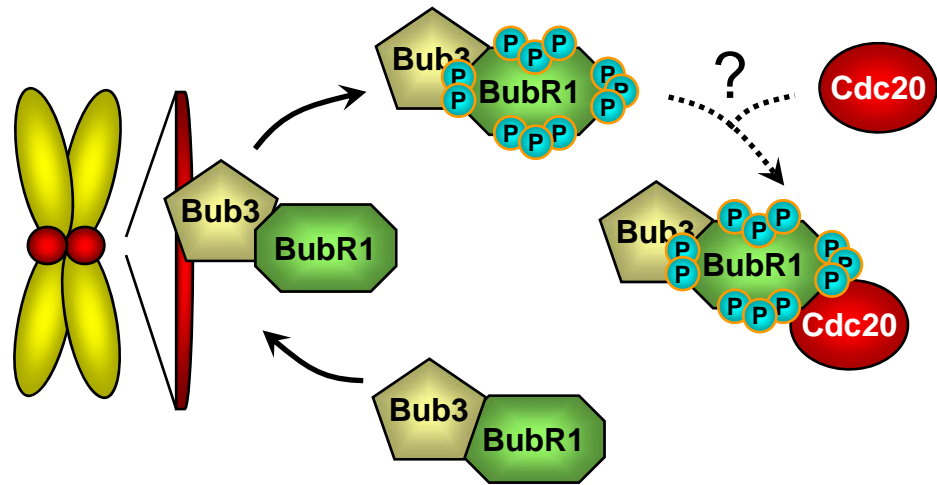


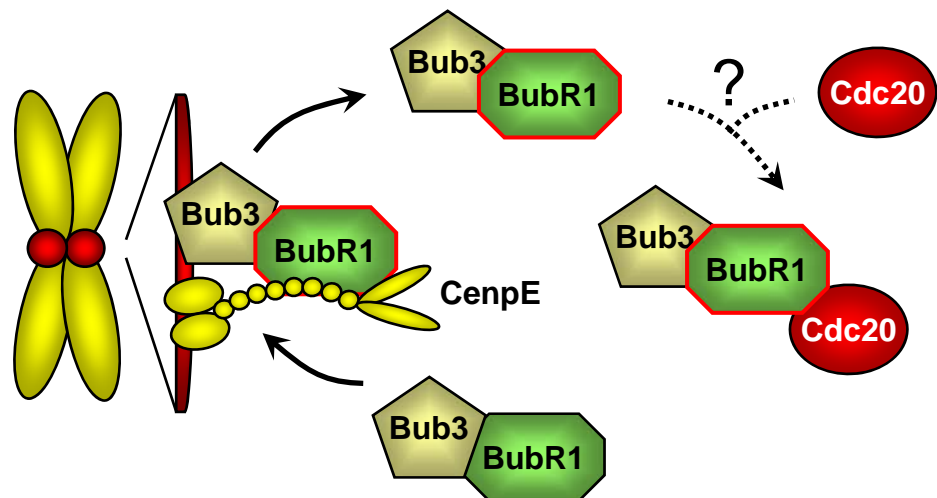
Figure 4. The function of BubR1 phosphorylation and kinase activity are not well understood.

(A) BubR1 localizes to kinetochores, where it is phosphorylated in mitosis. However, the relevance of BubR1 phosphorylation for its checkpoint functions, such as Cdc20 binding, is not known. (B) BubR1 kinase activity is likewise activated upon its interaction with CenpE at the kinetochores, but the role of the kinase activity for the checkpoint is contentious.

A



B



CHAPTER 2.

Unattached Kinetochores Generate a Checkpoint Inhibitor Through the Catalytic Activation of Mad2

Anita Kulukian, Joo Seok Han, Don Cleveland

Abstract:

Premature anaphase onset is prevented by the mitotic checkpoint through production of a “wait anaphase” inhibitor(s) that blocks recognition of cyclin B and securin by Cdc20-activated APC/C, an E3 ubiquitin ligase which targets them for destruction. Using physiologically relevant levels of Mad2, Bub3, BubR1, and Cdc20, unattached kinetochores on purified chromosomes competent for Mad2 recruitment are demonstrated to amplify production of a Cdc20 specific inhibitor to both interphase and mitotic APC/C. Chromosomes are shown to catalytically activate Mad2, but not BubR1, to inhibit APC/C^{Cdc20}. Antibody inhibition of Mad1 and dimerization deficient Mad2 are used to demonstrate that the chromosome-produced inhibitor requires both recruitment of Mad2 by Mad1 stably bound at unattached kinetochores and dimerization competent Mad2. While BubR1 is shown to be required for full APC/C inhibition, no distinct role could be demonstrated for the

phosphorylation and kinase activity of BubR1. BubR1 and Mad2 involvement in APC/C inhibition is shown to be sequential, supporting a model in which immobilized Mad1/Mad2 at kinetochores provides a template for initial assembly of Mad2-Cdc20, with BubR1 acting downstream of Mad2 activation.

Introduction:

When a cell undergoes a round of division, it must partition its genetic content equally to its daughter cells. Errors in chromosome segregation produce aneuploidy, which is frequently accompanied by altered growth properties, including inviability or as a contributor to oncogenesis (Weaver et al., 2007). To ensure accurate segregation, the major cell cycle control mechanism in mitosis, the mitotic checkpoint (also known as the spindle assembly checkpoint), delays anaphase onset until all chromosomes have properly attached to spindle microtubules. The checkpoint-derived inhibitor(s) blocks premature destruction of key mitotic components. This is achieved by selectively inhibiting Cdc20 stimulated recognition of the mitotic regulators cyclin B and securin by a multisubunit E3 ubiquitin ligase, the Anaphase Promoting Complex/Cyclosome (APC/C). Checkpoint silencing and subsequent deactivation of the checkpoint arrest releases APC/C^{Cdc20} for ubiquitination of cyclin B and securin, with anaphase triggered by their subsequent degradation by the proteasome (reviewed recently in (Baker et al., 2007; Peters, 2006)). A Cdc20 related protein, Cdh1, replaces Cdc20 in maintaining APC/C activity late in mitosis and through G1 of the next cell cycle (Fang et al., 1998b; Kramer et al., 1998).

By correlating the timing of anaphase onset with spindle microtubule capture by the last unattached chromosome (Rieder et al., 1994), laser

ablation of the last unattached kinetochore (Rieder et al., 1995) and micromanipulation (Li and Nicklas, 1995), unattached kinetochores were first implicated as essential for generation of the wait anaphase inhibitor. A combination of genetics in yeast and molecular biological manipulations in mammalian cells and amphibian egg extracts identified several key proteins essential for mitotic checkpoint signaling, including Mad1, Mad2, Bub3, CENP-E, Zw10, Rod, and the kinases Mps1, Bub1 and BubR1 (reviewed by (Musacchio and Salmon, 2007)), each of which is at least transiently localized to unattached kinetochores during early mitosis. Inhibition of Cdc20 activation of APC/C has previously been attributed to Mad2 (Fang et al., 1998a) or BubR1 (Tang et al., 2001), both of which can bind Cdc20 directly and in so doing have been shown to reduce APC/C^{Cdc20} ubiquitination activity accordingly. Addition of Mad2 or BubR1 to the other enhances inhibition of APC/C^{Cdc20} over either added singly *in vitro* (Fang, 2002). A complex, named the Mitotic Checkpoint Complex (MCC) and proposed to be comprised of Mad2, BubR1, Bub3 and Cdc20, has been reported to inhibit APC/C much more potently than Mad2 alone (Sudakin et al., 2001). Evidence for a similar complex (with Mad3 replacing BubR1) has also been proposed to be present in budding yeast (Fraschini et al., 2001; Hardwick et al., 2000). However the existence of MCC complexes has been noted outside of mitosis (Sudakin et al., 2001) or in the absence of a functional kinetochore (Fraschini et al., 2001). The simplest view is that an interphase mechanism independent of the

kinetochore generates a pre-made inhibitor(s) of Cdc20 that requires Mad2 and BubR1 and whose half life sets a minimum time before anaphase onset (Meraldi et al., 2004)

Fluorescence recovery after photobleaching (FRAP) demonstrated that Mad2, BubR1, and Cdc20 cycle on and off kinetochores with rapid dynamics (Howell et al., 2000; Howell et al., 2004; Kallio et al., 2002; Shah et al., 2004). Additionally, APC/C subunits APC1, APC3/Cdc27, APC6/Cdc16, APC8/Cdc23, and APC10/Doc1 are at least partially localized onto unattached kinetochores (Acquaviva et al., 2004; Jorgensen et al., 1998; Kurasawa and Todokoro, 1999; Topper et al., 2002; Vigneron et al., 2004), supporting the possibility that one or more of its components are sensitized for checkpoint inhibition there (Acquaviva et al., 2004; Sudakin et al., 2001). Bub1, which FRAP has demonstrated to be stably bound at unattached kinetochores (Howell et al., 2004; Shah et al., 2004), can phosphorylate Cdc20, potentially sensitizing it for checkpoint inhibition (Tang et al., 2004).

A “template” model for kinetochore-dependent activation of Mad2, a modified version of the “two-state” model (De Antoni et al., 2005; Mapelli and Musacchio, 2007; Yu, 2006), has emerged from use of cultured cells and purified components in the absence of chromosomes and the discovery that Mad2 can undergo a large conformational change in which its carboxy terminal “seatbelt” domain encloses either Mad1 or Cdc20, thereby converting the initial Mad2 from an “open” (or “N1”) to a “closed” (or “N2”) conformation

(Luo et al., 2000; Luo et al., 2002; Luo et al., 2004; Sironi et al., 2002; Sironi et al., 2001). The closed conformation has been proposed to sequester Cdc20 from binding to and activating APC/C for recognition of cyclin B through direct capture of Cdc20 by Mad2 (Fang et al., 1998a; Luo et al., 2004; Tang et al., 2001). The carboxy terminal domain of Mad1 has been shown to directly bind a molecule of Mad2 in the closed conformation (De Antoni et al., 2005; Luo et al., 2002; Luo et al., 2004; Sironi et al., 2002; Sironi et al., 2001). FRAP within PTK2 cells has revealed that Mad1 at kinetochores, presumably bound to Mad2, is non-exchangeable, while two equally sized pools of kinetochore-associated Mad2 either cycle on and off rapidly (within a few seconds) or are more stably bound (Shah et al., 2004). Mad2 mutants impaired in dimerization were subsequently shown to be unable to support either mitotic checkpoint signaling *in vivo* (De Antoni et al., 2005; Mapelli et al., 2006; Nezi et al., 2006) or capture of Cdc20 (using a 27 amino acid peptide to mimic Cdc20) facilitated by Mad2 in a complex with a 233 amino acid Mad2-binding fragment of Mad1 (De Antoni et al., 2005). *In vitro* FRAP was used to demonstrate recruitment of a rapidly exchangeable Mad2 by a second stably associated Mad2 bound in the closed conformation to full length or a fragment of Mad1. There was, however, no evidence for facilitating conversion of soluble Mad2 to Mad2-Cdc20 and the rate of Mad2 dissociation from the immobilized Mad1/Mad2 was independent of the Cdc20 peptide (Vink et al., 2006).

Despite these preceding discoveries, neither the identity(ies) of the

checkpoint-derived anaphase inhibitor nor how unattached kinetochores participate in its production is established. To pose a direct test of the role of the kinetochore in the production of an APC/C^{Cdc20} inhibitor, we have now used purified components to reconstruct unattached kinetochore dependent signaling and APC/C^{Cdc20} inhibitor production.

Results:**Mad2 recruitment to unattached kinetochores on purified chromosomes.**

To reconstruct kinetochore-mediated mitotic checkpoint signaling with all purified components, differential sedimentation including successive sucrose gradients was used to isolate chromosomes with unattached kinetochores from nocodazole arrested, mitotic HeLa cells stably expressing histone H2B-YFP (Figure 5A). This yielded morphologically intact, condensed chromosomes, as observed by YFP fluorescence of unfixed chromosomes (Figure 5B). To assess the protein composition, chromosomes isolated after the first and second gradients were pelleted by a final centrifugation step (Figure 6A). Tubulin was reduced to less than 1/40th of its concentration in the initial cell extracts (Figure 6C), while histones (including H2B-YFP and the mitotic-specific phosphorylated histone H3) and kinetochore-associated kinesin-like motor protein CENP-E were nearly quantitatively retained (Figure 5B). Components previously reported by FRAP to be stably bound to unattached kinetochores, including Bub1 and Mad1 (Howell et al., 2004; Shah et al., 2004), were also retained, as was Mps1 and a proportion of the inner centromere protein Aurora B (Figure 5B).

Using anti-centromere antisera (ACA) and immunofluorescent staining of fixed, DAPI stained chromosomes to identify centromeres/kinetochores on purified chromosomes, Bub1, Mad1 and CENP-E were demonstrated to

remain kinetochore-bound (Figure 7). Components for which FRAP had revealed rapid cycling, including BubR1, Bub3, and Cdc20 (Howell et al., 2000; Howell et al., 2004; Kallio et al., 2002; Shah et al., 2004), were undetectable by immunoblotting of the purified chromosome fractions (Figure 6B) (their levels in purified chromosomes were all $\leq 1.1\%$ of the cytosolic levels – Figure 8). Although a portion of Mad2 is known to be stably bound at an unattached kinetochore for at least two minutes *in vivo* (Shah et al, 2004), Mad2 nearly completely dissociated from most kinetochores during the 7 hours required for chromosome isolation (Figure 6B), with faint levels detectable by immunofluorescence remaining only on a minority (20.1%) of isolated chromosomes (Figure 7).

Competence of kinetochores on the purified chromosomes for recruitment of Mad2 was tested by addition of purified, bacterially produced Mad2 isolated under conditions promoting primarily the retention of the open monomeric conformation (Luo et al., 2004; Mapelli and Musacchio, 2007), the conformation thought to be representative of cytosolic Mad2 (Luo et al., 2004). After covalent ligation of rhodamine (Figure 9A,B) and addition to purified chromosomes, rhodamine-Mad2 bound to unattached kinetochores of the majority of chromosomes (Figure 9C; see also Figure 23,27 for quantification). At least a portion of this Mad2 binding was mediated through kinetochore associated Mad1, as demonstrated by partial blocking even by brief pre-incubation of the chromosomes with an antibody against Mad1 (Figure 27).

Generation by unattached kinetochores of an APC/C-Cdc20 inhibitor.

To determine how unattached kinetochores produce a mitotic checkpoint signal, we established an *in vitro* assay for Cdc20-stimulated ubiquitination by APC/C (Figure 10D). Human homologues of Mad2 and BubR1, as well as Bub3, Cdc20, and the APC/C G1-specific activator Cdh1 were produced in bacteria or in insect cells using baculovirus and purified (Figure 10A). The APC/C complex was immunoprecipitated from *Xenopus* interphase (Figure 10C) or mitotic (Figure 13) extracts with an antibody against the Cdc27 subunit. For mitotic APC/C, immunoblotting for Cdc27 revealed a slowed mobility of Cdc27 both in the initial mitotic extract and after isolation (Figure 13B), a shift known to reflect mitotic phosphorylation (Kraft et al., 2003). Addition of Cdc20 to either APC/C activated the ubiquitination of cyclin B₁₋₁₀₂ (Figure 11), which could be quantified either by the presence of slower mobility cyclin B species (Figure 11B, top) or the intensity of the remaining un-ubiquitinated cyclin B pool (Figure 11B, bottom). [Note that it is possible that only a fraction of each recombinant protein, especially Cdc20 (which has a reported requirement for the CCT chaperone for its proper folding (Camasses et al., 2003)), is fully active. With this in mind and the potential of variability between different purifications, in all experiments below we have relied only on direct comparisons within a contemporaneous series of ubiquitination assays.]

To assess the basal inhibition of APC/C^{Cdc20} by our purified checkpoint proteins, ubiquitination activity was initially assayed in the presence of equal molar amounts of Mad2, BubR1, or Bub3 to approximate the relative *in vivo* stoichiometries (Figure 11). [The molar level of Bub3 *in vivo* is actually about twice the levels of the other proteins, but it is a stoichiometric binding partner for both BubR1 and Bub1 (Taylor et al., 1998).] While under these conditions addition of Mad2, Bub3 or BubR1 alone did not significantly inhibit ubiquitination of cyclin B, the combination of Mad2 and BubR1 did, independent of Bub3 (Figure 11C). Inhibition was selective for Cdc20-mediated activation of APC/C, as similar addition to APC/C^{Cdh1} left ubiquitination activity undiminished (Figure 14A,B). BubR1 was a significantly better inhibitor of Cdc20 activation of APC/C: a >10 fold molar excess of Mad2 over Cdc20 was necessary to achieve >50% inhibition, whereas 10 times less of BubR1 with or without Bub3 was required for equivalent APC/C^{Cdc20} inhibition (Figure 13,15B). Nevertheless, there was synergism between Bub3/BubR1 and Mad2. Inhibition by the combination of Bub3/BubR1 and Mad2 was greater than the additive effect of each alone. Suppression of cyclin B ubiquitination by BubR1 was enhanced by Mad2 such that a quarter of the amount of BubR1 was required for equivalent inhibition of Cdc20 in the presence of Mad2 (Figure 12B, lane 8 vs 13), even though the same amount of Mad2 alone produced no observable inhibition (Figure 12B, lane 6 vs 13). These results were similar to an earlier report (Fang, 2002) demonstrating that

Mad2 and Bub3/BubR1 cooperate to inhibit Cdc20 even in the absence of unattached kinetochores.

Addition to APC/C^{Cdc20} of increasing levels of Mad2 alone produced dose dependent inhibition of cyclin B ubiquitination (Figure 15A, lanes 3-6), but even a 20 fold excess of Mad2 over Cdc20 produced only 50% inhibition. Although purified chromosomes alone minimally inhibited APC/C^{Cdc20}, addition of them to a concentration approximating ten unattached kinetochores per cell volume amplified the inhibition produced at all concentrations tested of added Mad2 (Figure 15A, lanes 8-11). In the absence of chromosomes, BubR1 yielded comparable inhibition of APC/C^{Cdc20} at 10-20 fold lower levels than required for Mad2. Unlike Mad2, addition of chromosomes to BubR1 had no effect on the corresponding inhibition of APC/C^{Cdc20} (Figure 15B). Inhibition of mitotic (Figure 13C) or interphase (Figure 13C,15C) APC/C^{Cdc20} by a combination of Mad2, Bub3 and BubR1 was seen at all concentrations. For example, although equimolar additions of Mad2, Bub3, BubR1 produced only very partial inhibition of Cdc20-dependent activation of APC/C, unattached kinetochores produced equivalent inhibition at 10 fold lower levels of added Mad2 and BubR1/Bub3 (Figure 15C, compare lanes 3 versus 11 and 14 versus 22). Moreover, chromosome-dependent inhibitory activity was selective for Cdc20, as APC/C^{Cdh1} ubiquitination of cyclin B was unaffected even at maximal doses of Bub3/BubR1, Mad2 and chromosomes (Figure 14C,D). Chromosome amplification of APC/C^{Cdc20} inhibition was greatest at

the lowest concentrations of added Mad2 and BubR1/Bub3, with maximal inhibition 35 fold that seen in the absence of chromosomes (Figure 15C, 0.1x concentration). This was achieved by a chromosome-dependent effect on Mad2 (see also Figure 29 below).

BubR1 phosphorylation and kinase activity are not required for its inhibition of APC/C.

Recombinant purified BubR1 lacks the retarded mobility characteristic of its checkpoint active hyper-phosphorylated mitotic state and evident of trace BubR1 remaining on purified chromosomes (Figure 8A). However, immunoblotting with an antibody specific for Serine-670, a residue specifically phosphorylated in mitosis (Elowe et al., 2007), demonstrated that BubR1 purified from insect cells was phosphorylated at least on this particular site (Figure 16, lane 3). The phosphorylation could be removed with λ -Phosphatase treatment (Figure 16, lane 5). Incubation with chromosomes did not increase the degree of phosphorylation at this site (Figure 16, lane 8,10).

As a more thorough approach for addressing whether BubR1 phosphorylation was required for its role in APC/C inhibition, wild type BubR1 was compared to a mutant which could not be phosphorylated (BubR1^{Ph11A}). The mitosis-specific phosphorylation sites were mapped by mass spectrometry and the ten serines and one threonine identified (Figure 17B) were mutated to alanines (Geert Kops, unpublished). The mutated protein was

purified (Figure 17A) and compared to wild type BubR1 inhibition of APC/C in ubiquitination assays in the absence of chromosomes. Surprisingly, non-phosphorylatable BubR1 was a more potent inhibitor of the APC/C than unmodified BubR1 (Figure 17C). Addition of chromosomes to BubR1^{Ph11A} slightly reduced its potency (Figure 17D).

To test the requirement of BubR1 kinase activity for APC/C inhibition, ubiquitination assays in the presence of BubR1 were supplemented with ATP. Preliminary experiments (n=1) demonstrated that addition of ATP to stimulate BubR1 kinase activity did not improve its potency as an APC/C inhibitor (Figure 18A) in the absence of unattached kinetochores. When compared to kinase dead mutant of BubR1 (Figure 19A), which is mutated at Lysine 795 to an arginine, wild type BubR1 was a slightly weaker inhibitor of APC/C^{Cdc20} (Figure 19B). In the presence of unattached kinetochores, while BubR1^{K795R} activity remained unperturbed with ATP addition (Figure 19C), BubR1^{wt} increased in potency only when added at lower concentrations (Figure 18B). A more thorough investigation is needed for conclusive interpretation of these results.

Catalytic production by unattached kinetochores of a Mad2 Cdc20 inhibitor.

A central unresolved question is whether unattached kinetochores act catalytically in the production of a Cdc20 inhibitor. A central requirement of a

catalytic model would be for unattached kinetochores to accelerate the rate of production of a Cdc20 inhibitor, while an extended incubation without kinetochores could ultimately yield comparable inhibition mediated by an uncatalyzed, spontaneous process. Addition of chromosomes to checkpoint inhibitors produced maximal inhibition of APC/C within 30 minutes, while in the absence of chromosomes, APC/C inhibition gradually increased over a time span of one hour without reaching a steady state (Figure 20). To further test the catalytic model, Mad2 (the principal molecule on which kinetochores seem to act – Figure 15A) and Cdc20 were added to a level sufficient to yield 40% inhibition after extended incubation without addition of chromosomes. In the absence of chromosomes, a linear increase in inhibition of Cdc20 was produced over the first 120 minutes, ultimately plateauing at about 40% inhibition by 4 hours (Figure 21). As required for chromosome-dependent catalysis, the presence of a concentration of chromosomes corresponding to 10 unattached kinetochores per cell accelerated the initial rate of inhibitor production 8 fold (initial slopes of 2.5 versus 0.3 % inhibition/min in the presence and absence of chromosomes, respectively) (Figure 21), with the final level of inhibition similar to that produced spontaneously.

Mad2 dimerization is required for kinetochore amplification of Cdc20 inhibition.

To determine if recruitment of Mad2 to unattached kinetochores via Mad1 (Chen et al., 1999; Chen et al., 1998; Luo et al., 2002; Sironi et al., 2001), and Mad2 binding directly to Cdc20 were required for chromosome amplification of APC/C^{Cdc20} inhibition, APC/C activity assays in the presence of added chromosomes were performed with wild type Mad2 or a Mad2 mutant that can bind to kinetochore-associated Mad1 or Cdc20, but is incompetent for dimerization onto Mad1/Mad2 complexes (Figure 25). For this, we chose the Mad2^{RQ} mutant [carrying the two amino acid substitution R133E, Q134A], previously demonstrated as incapable of supporting full mitotic checkpoint function in vivo (De Antoni et al., 2005) (Figure 22). Incubation of rhodamine-labeled Mad2^{RQ} (Figure 23) with purified chromosomes yielded Mad2 localization to kinetochores with equal frequency as wild type Mad2 (Figure 23C), yet with approximately half the intensity (Figure 23D). Thus, Mad2^{RQ} bound directly to Mad1 at kinetochores mostly depleted of endogenous Mad2 (Figure 6B), yet was unable to recruit a second Mad2 molecule to form Mad2 dimers. In the absence of chromosomes, Mad2^{RQ} inhibited Cdc20-stimulated APC/C ubiquitination almost as well as wild type Mad2 did (Figure 24). This Cdc20 inhibition required direct binding of Mad2 to Cdc20: Mad2 mutants missing the carboxy-terminal 10 amino acids (Mad2^{ΔC} or Mad2^{RQ-ΔC}; Figure 22,23) that are required for Cdc20 binding (Luo et al., 2000) did not inhibit APC/C^{Cdc20} at any concentration (Figure 24).

In contrast to wild type Mad2, the dimerization deficient Mad2^{RQ} was incapable of supporting chromosomal amplification of APC/C inhibition at any added concentration (Figure 25). Moreover, when tested for synergy with Bub3/BubR1 and chromosomes in inhibiting APC/C^{Cdc20}, Mad2^{RQ} was much less efficient than wild type Mad2 (Figure 26). This finding also strongly suggests that kinetochores act almost exclusively on Mad2, not BubR1, to amplify generation of a Cdc20 inhibitor.

Kinetochores-bound Mad1 is required for chromosome-mediated amplification of a Cdc20 inhibitor.

To further test the Mad1 role in the chromosome-dependent amplification of an APC/C^{Cdc20} inhibitor, a Mad1 antibody raised against the region that spans the Mad2 binding domain was added to isolated chromosomes in an effort to inhibit its function at kinetochores (Figure 27,28). This substantially reduced Mad2 recruitment to kinetochores (Figure 27), as anticipated. In the absence of chromosomes or Mad2, Mad1 antibody had no effect on APC/C^{Cdc20} ubiquitination (Figure 28). In the presence of a five fold excess of Mad2 (the concentration chosen for the greatest inhibitory difference upon addition of chromosomes), chromosomes amplified APC/C^{Cdc20} inhibition seven fold, but this amplification was almost eliminated by Mad1 antibody addition (Figure 28). Thus, Mad1 acting at kinetochores is required both for

kinetochore recruitment of Mad2 and the chromosome-mediated enhancement of inhibition of APC/C^{Cdc20}.

Unattached kinetochores act on Mad2 for amplifying a Cdc20 inhibitor.

Catalytic amplification by chromosomes of a Cdc20 inhibitor was maximal at the lowest Mad2 concentrations (Figure 15A,21) and required dimerization competent Mad2 interacting with kinetochore bound Mad1 (Figure 25,26), suggesting a model in which unattached kinetochores act catalytically on Mad2, but not BubR1, to accelerate the rate of production of an initial Mad2-Cdc20 inhibitor. To test if unattached kinetochores act directly on Mad2, chromosomes and Cdc20 were incubated with Mad2, the chromosomes were then removed, BubR1/Bub3 was added, and finally APC/C was added and assayed for ubiquitination of cyclin B (Figure 29). Comparable to the stimulation of inhibition of Cdc20 that was found when all components were incubated together with chromosomes, a 4 fold stimulation of inhibitory activity was generated when only Mad2 and Cdc20 were co-incubated with chromosomes (Figure 29C). Thus, amplification of a Cdc20 inhibitor does not require unattached kinetochores acting directly on either APC/C or BubR1/Bub3, but rather by direct interaction with Mad2 and/or Cdc20. BubR1/Bub3 is thus suggested to act downstream of Mad2.

Discussion:**Unattached kinetochores catalyze production of a “wait anaphase” inhibitor.**

While unattached kinetochores have been widely inferred to be the source of a “wait anaphase” mitotic checkpoint inhibitor, we have now demonstrated that kinetochores can, in fact, catalyze production of a Mad2-Cdc20 inhibitor, significantly accelerating the initial rate of its production. More importantly, at physiologically relevant concentrations of Mad2, Bub3/BubR1 or their combination, chromosomes catalyzed production of Cdc20 inhibition of cyclin B recognition by APC/C by at least 8 fold relative to inhibitors formed spontaneously in the absence of chromosomes. Unattached kinetochores do this by acting directly only on Mad2, catalyzing inhibition of Cdc20 by Mad2 alone, but leaving unaffected inhibition by Bub3/BubR1 in the absence of Mad2. The actual *in vivo* effect is likely to be much greater than what we have observed *in vitro*, since chromosome purification resulted in partial loss of signaling molecules from kinetochores, including a proportion of Mad1 and kinases that include Bub1, BubR1 and Aurora B.

A Kinetochose template that acts on Mad2, but not on BubR1.

Preceding work has supported a “template” model for recruitment of soluble, open Mad2 by immobilized, kinetochose-bound Mad1/Mad2 and

conversion at the kinetochore into a form with its seatbelt domain poised for Cdc20 capture (De Antoni et al., 2005; Mapelli et al., 2006; Shah et al., 2004; Vink et al., 2006). The current template model has not, however, included a role for the essential mitotic checkpoint protein BubR1, despite its known binding to Cdc20 with a higher affinity than does Mad2 (Fang, 2002; Tang et al., 2001). We have directly tested aspects of the template model. Chromosome amplification of Cdc20 inhibition required Mad1 recruitment of Mad2 to kinetochores and dimerization competent Mad2 (Figure 26,28), thereby providing a direct demonstration that a Mad1:Mad2 core complex recruits and converts soluble “inactive” Mad2 into a more potent inhibitor of Cdc20. At least part of this is from action of kinetochores on Mad2.

BubR1 localizes to kinetochores with its binding partner Bub3 (Taylor et al., 1998), where it is reported to be phosphorylated by Plk1/Pix1/Polo (Elowe et al., 2007; Rancati et al., 2005; Wong and Fang, 2007), Cdk1 (Wong and Fang, 2007), and/or Aurora B/Ipl1 (King et al., 2007; Morrow et al., 2005; Rancati et al., 2005) in a Mad1 dependent manner (Chen, 2002). Yet, Aurora B activity is neither essential for BubR1 association with the APC/C (Morrow et al., 2005) nor are securin levels affected in cells lacking Plk1 (van Vugt et al., 2004). Likewise, a non-phosphorylatable mutant of BubR1 at the Cdk1 site utilized by Plk1 for docking is equally able to inhibit ubiquitination by APC/C *in vitro* (Wong and Fang, 2007). Finally, while BubR1 kinase activity is stimulated upon its interaction with CENP-E at the kinetochores (Mao et al., 2003), it is

not required for kinetochore-independent inhibition of APC/C *in vitro* (Tang et al., 2001). While we by no means exclude a kinetochore-dependent function of BubR1 for roles in microtubule attachment and chromosome alignment (Ditchfield et al., 2003; Elowe et al., 2007; Lampson and Kapoor, 2005; Matsumura et al., 2007) or for further amplification of a kinetochore derived signal (Mao et al., 2003), kinetochore-mediated enhancement of Cdc20 inhibition did not require BubR1 localization to or contact with kinetochores. We conclude that kinetochore localization is not required for at least a portion of BubR1's role in the catalytic production of a mitotic checkpoint inhibitor.

Regardless of whether its phosphorylation or kinase activity are required for APC/C inhibition, BubR1 does more potently inhibit the APC/C than Mad2 and is required for its maximal inhibition (Figure 15B). Kinetochores may directly act on Mad2, but without BubR1, APC/C activity cannot be fully restrained, even over an extended period of time (Figure 15C). Conversely, BubR1 itself is dependent upon Mad2 for complete inhibition of the APC/C. Evidence presented in this chapter is suggestive of a role for BubR1 that is downstream of kinetochore activation of Mad2, a point that will be further explored in Chapter 3.

Materials and Methods:

Protein Purification. Recombinant proteins His-E1, His-UbcH10, His-Mad2^{wt}, His-Mad2^{RQ}, His-Mad2^{ΔC}, His-Mad2^{RQ-ΔC}, and His-Myc-Cyclin B₁₋₁₀₂ were purified from bacteria after induction while His-Cdc20, His-Cdh1, and His-Bub3 were purified from SF9 cells post baculovirus infection using a HIS tag purification as previously described (Tang and Yu, 2004). Human BubR1 cDNA was cloned into pFastBac1 expression plasmid together with GST tag-PreScission protease cleavage sequence at the N terminus and the recombinant plasmid was used for making baculovirus encoding GST-BubR1. GST-BubR1 was expressed from Hi5 insect cells infected with the recombinant baculovirus and affinity purified over glutathione sepharose beads. BubR1 was eluted by PreScission protease digestion to cleave the GST tag. Monomeric BubR1 was separated from oligomeric species on a gel filtration column. APC/C was immunoprecipitated from *Xenopus* egg extracts cycled into interphase with calcium addition, or from checkpoint active mitotic extracts post sperm and nocodazole addition.

Chromosome Purification. HeLa cells stably transfected with YFP-H2B were treated with 50ng/ml colcemid for 16 hours. Mitotic cells were collected by shake-off and subjected to hypotonic conditions in modified PME buffer (MPME; 5 mM PIPES pH 7.2, 10 mM NaCl, 5 mM MgCl₂, 0.5 mM EGTA, and 2 mM EDTA). The swollen cells were resuspended in 10x volume of MPME

buffer supplemented with 10 µg/ml LPC, 0.5 mM spermine, 1 mM Spermidine, 10 mM NaF, 1 mM NaVO₄, along with 1 mM PMSF and 0.1% digitonin. The cells were disrupted using a dounce homogenizer to produce an initial lysate (total lysate). The lysate was centrifuged briefly at 900 x g for 1 min to pellet intact nuclei and cell debris (cleared lysate). The NaCl concentration of the solution was subsequently elevated to 100 mM (hsMPME), and the lysate was placed over a sucrose step gradient (30%-40%-50%-60%) prepared with supplemented hsMPME and centrifuged for 15 minutes at 5000 x g. The top layers were removed by suction and the flocculent white material at the 40%-50% and 50%-60% interface containing the chromosomes was harvested with a Pasteur pipette (1st sucrose gradient). The chromosomes were then washed in 8 volumes of supplemented hsMPME buffer, sedimented for 15 min at 2900 x g, suspended in supplemented hsMPME, placed over a second sucrose gradient, and reharvested as described above. The chromosomes were washed a second time and resuspended in approximately 10 volumes chromosome storage buffer (hsMPME containing 50% sucrose, 0.5 mM spermine, 10 mM NaF, 1 mM NaVO₄, 10 µg/ml LPC), thereby producing 2nd gradient chromosomes. The chromosomes were aliquoted, frozen rapidly in liquid nitrogen, and stored at -80°C.

APC/C Ubiquitination / Depletion Assays. APC/C ubiquitination assays were performed as previously described (Tang and Yu, 2004), utilizing different anti-Cdc27 antibodies depending on whether ubiquitination (Fang et

al., 1998a) or depletion of cyclin B was measured (Tugendreich et al., 1995). To analyze the depletion assays, film exposures within a nearly linear range based on an internal loading gradient of inactive APC/C were selected. The intensity of bands corresponding to unubiquitinated cyclin B on immunoblots was quantified using NIH Image J software. The intensities of each lane were normalized against inactive APC/C (no Cdc20 added – 0%) and fully active APC/C (with Cdc20 added, no inhibitors added – 100%). Each depletion assay was repeated at least in triplicate and the average represented in graph format, with bars representing standard error of the mean.

Immunofluorescence of Chromosomes. Purified chromosomes were fixed in formaldehyde, placed over a 33% glycerol cushion, and sedimented onto coverslips by centrifugation for 20 min at 5500 x g. The chromosomes were subsequently fixed in ice cold methanol and processed as described (Weaver et al., 2003). The coverslips were stained with the following antibodies: Hpx antibody to CENP-E (Brown et al., 1996); sheep SB1 antibody to Bub1 (Taylor et al., 2001); BB3-8 antibody to Mad1 (De Antoni et al., 2005); rabbit anti-Mad2 (Kops et al., 2004) and ACA sera for identifying centromeres (Antibodies Inc.) Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Fixed and stained chromosomes were mounted with Prolong antifade reagent (Invitrogen). Chromosomes were imaged using a DeltaVision deconvolution microscope (Applied Precision). Optical sections were taken at 0.15 intervals and deconvolved using SoftWoRx software (Applied Precision).

The images were generated by projecting the sum of the stack of deconvolved images. Images were processed after equivalent scaling.

Labeling of Mad2 and its Localization onto Kinetochores. Recombinant Mad2 and mutants of it were fluorescently labeled using FluoReporter Rhodamine Red-X Protein Labeling Kit by Molecular Probes. Equivalently labeled batches of Mad2 were selected. Chromosomes were incubated with 480 nM rhodamine-labeled Mad2 fractions for 1hr at room temperature. For Mad1 antibody blocking, the chromosomes were first incubated with the Mad1 antibody for 10 min at room temperature and subsequently with the labeled Mad2. The chromosomes were treated for imaging as described above. Kinetochores fluorescence was quantified using the average intensity of traced kinetochore shape, as determined by ACA staining, using MetaMorph Imaging software (Molecular Devices).

The text of Chapter 2 and 3, in part and with modifications, will be published in Developmental Cell, 2009, as a single manuscript. I was the primary researcher and author of this work. Joo Seok Han also contributed to this work. Don Cleveland directed and supervised the research that forms the basis of these chapters.

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Figure 5. Purified chromosomes maintain structural integrity.

(A) Schematic of chromosome purification from mitotic HeLa cells stably transfected with the YFP-H2B histone. Cells were collected after 16 hours in 50 ng/ml colcemid and lysed by douncing in hypotonic buffer. Cell debris was removed by pelleting and the chromosome containing supernatant was fractionated by sequential sucrose gradients. **(B)** Morphology of purified chromosomes detected by fluorescence of YFP-H2B on coverslips without fixation.

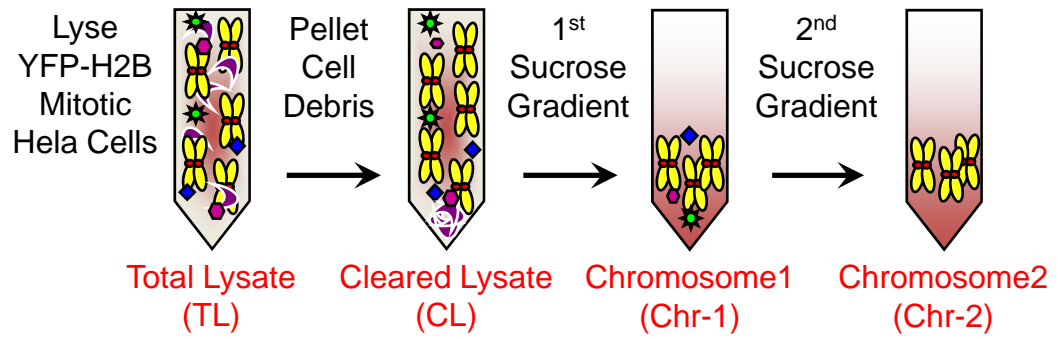
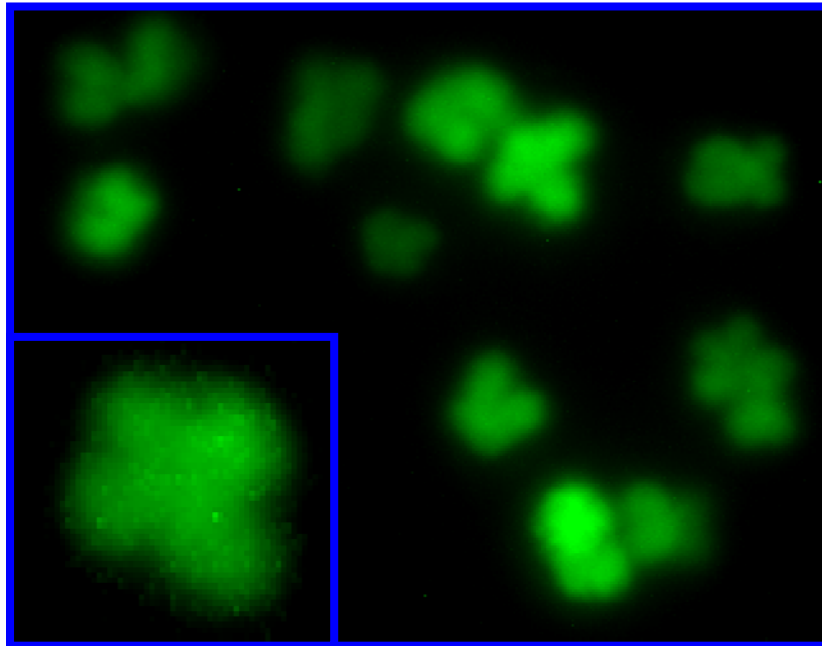
A**B**

Figure 6. Purified chromosomes retain stably bound components, but are depleted of contaminants and transiently bound components.

(A) Purified chromosomes were subjected to centrifugation to separate the chromatin fraction from the supernatant. **(B)** Protein constituents of purified chromosomes assessed by immunoblotting after centrifugation of each fraction to pellet chromosomes. The stably bound chromosome components included Mad1, Bub1, CENP-E, Aurora B, and Mps1, while Bub3, Mad2, Cdc20, and p31-Comet were nearly absent. **(C)** Assessment of tubulin levels remaining in purified chromosomes compared to a dilution series of the initial cellular input. Less than 1/40th of the initial tubulin level remains.

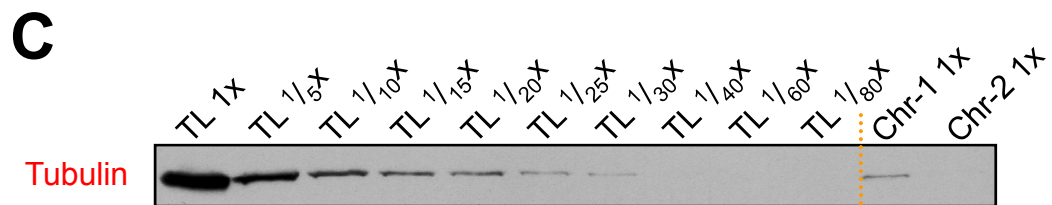
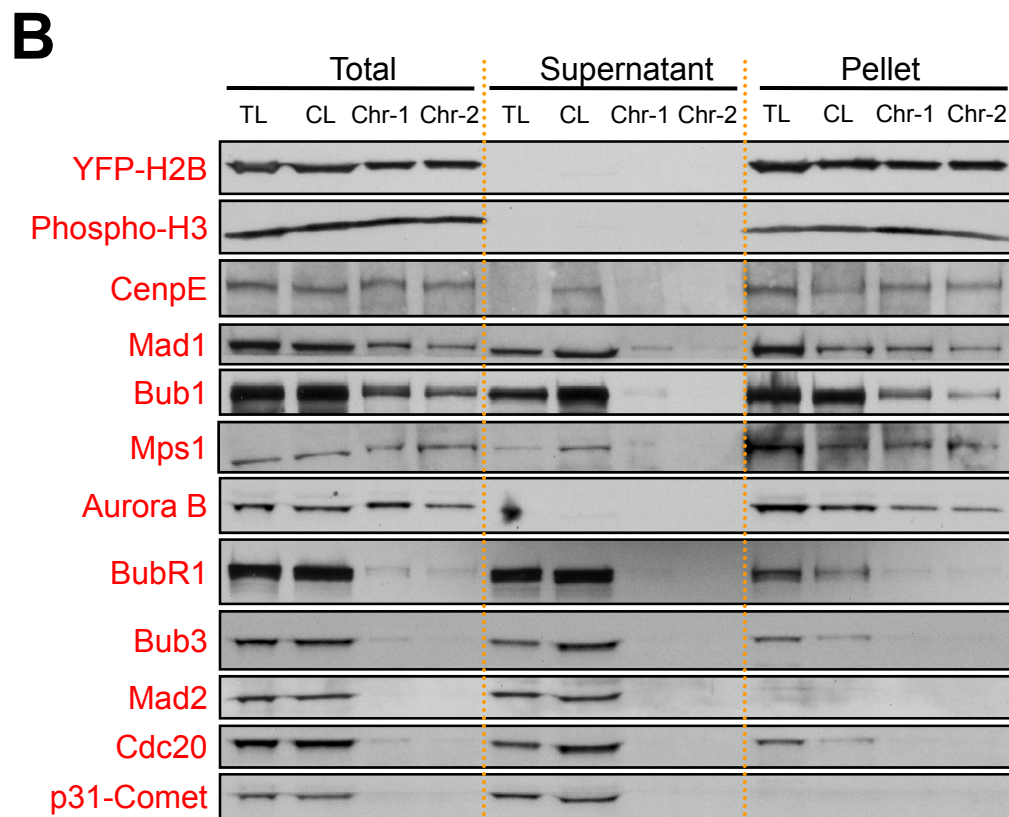
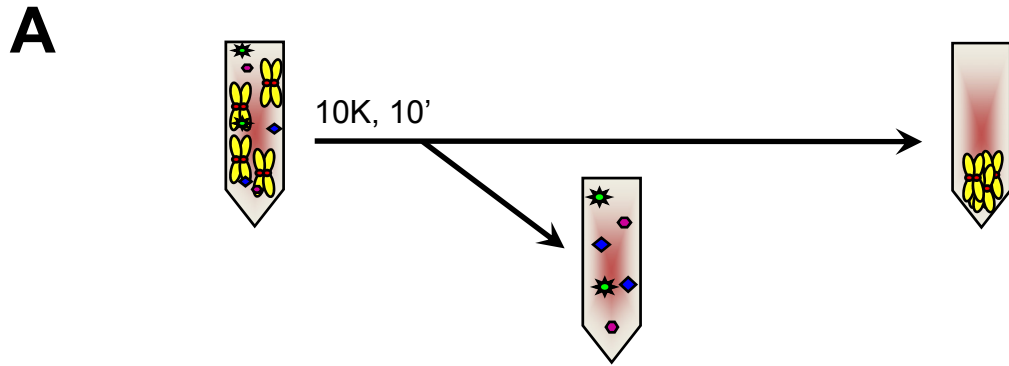


Figure 7. Stably bound checkpoint proteins are localized to unattached kinetochores of purified chromosomes.

Indirect immunofluorescence revealed that kinetochores on isolated chromosomes retained Mad1, Bub1, and CENP-E, while Mad2 was undetectable or weakly detectable. (Blue) Chromosomes stained with DAPI; (Green) Anticentromere (ACA) antibodies; (right panel) merged image.

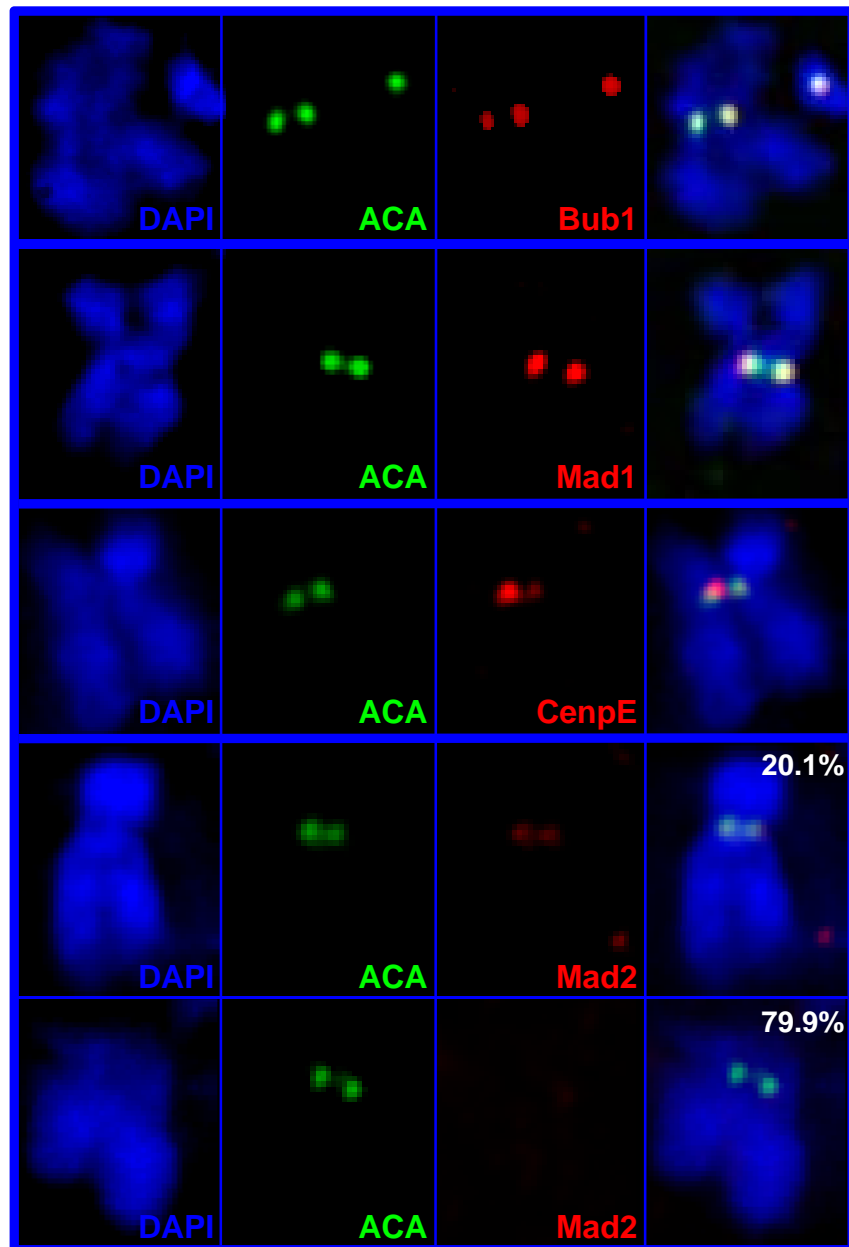
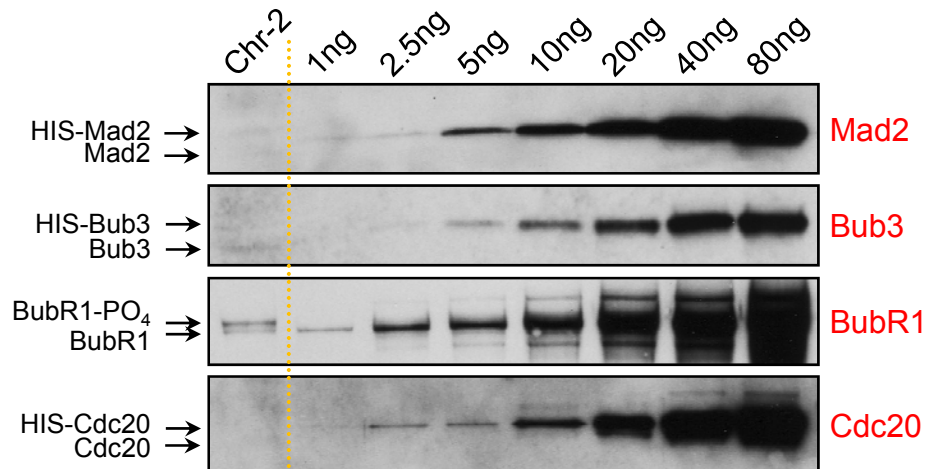
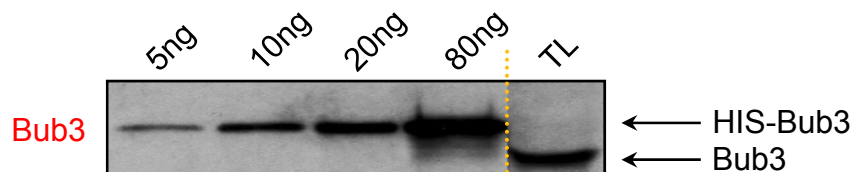


Figure 8. Purification of mitotic chromosomes reduces cytosolic contamination to ~1% the original cellular concentrations.

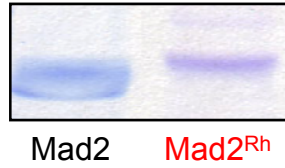
(A) The amount of Mad2, BubR1, Bub3, and Cdc20 remaining on purified chromosomes (Chr-2) was determined by immunoblot comparison with a dilution series of by a purified recombinant proteins. Protein concentrations of the purified recombinant proteins were determined by Coomassie staining and comparison to known amounts of bovine albumin. Mobility shifts due to protein tags or phosphorylation are as noted. **(B)** The cellular concentration of Bub3 was determined by loading mitotic cellular lysate (TL) (from 3.5×10^5 cells against a concentration gradient of purified Bub3 protein. **(C)** Table of concentrations and numbers of molecules of Cdc20, Mad2, BubR1 and Bub3 in whole cell extracts and on isolated chromosomes. Calculations were based upon the measurement that an individual cell volume is 9.1 pL, as calculated from the observation that a cell pellet containing 2.5×10^8 cells had a volume of 2.3 mL. Numbers for corresponding cellular and purified chromosome concentrations of Cdc20, Mad2, and BubR1 were previously determined (Tang et al., 2001).

A**B****C**

	Estimated cellular concentration (nM)	Molecules per cell	Molecules remaining on chromosomes per cell volume	Percentage of initial cellular concentration
Mad2	120	66,000	<710	<1.1%
Bub3	195	107,000	1000	0.9%
BubR1	90	50,000	220	0.4%
Cdc20	100	55,000	<330	<0.6%

Figure 9. Recombinant Mad2 localizes to kinetochores of purified chromosomes.

(A) Purified recombinant Mad2 before and after covalently labeling with rhodamine, assessed by Coomassie staining. **(B)** Rhodamine labeled recombinant Mad2 retains ability to inhibit Cdc20 activation of APC/C, as assessed by the presence of ubiquitin conjugated cyclin B species. **(C)** Purified chromosomes were incubated with rhodamine-labeled Mad2, fixed, stained for (blue) DAPI and (green) ACA, and imaged by deconvolution microscopy.

A**B**

iAPC/C	+	+	+
Cdc20	-	+	+
Mad2 ^{Rh}	-	-	10x

Cyclin B - Ub
Conjugates

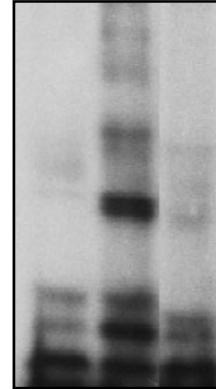
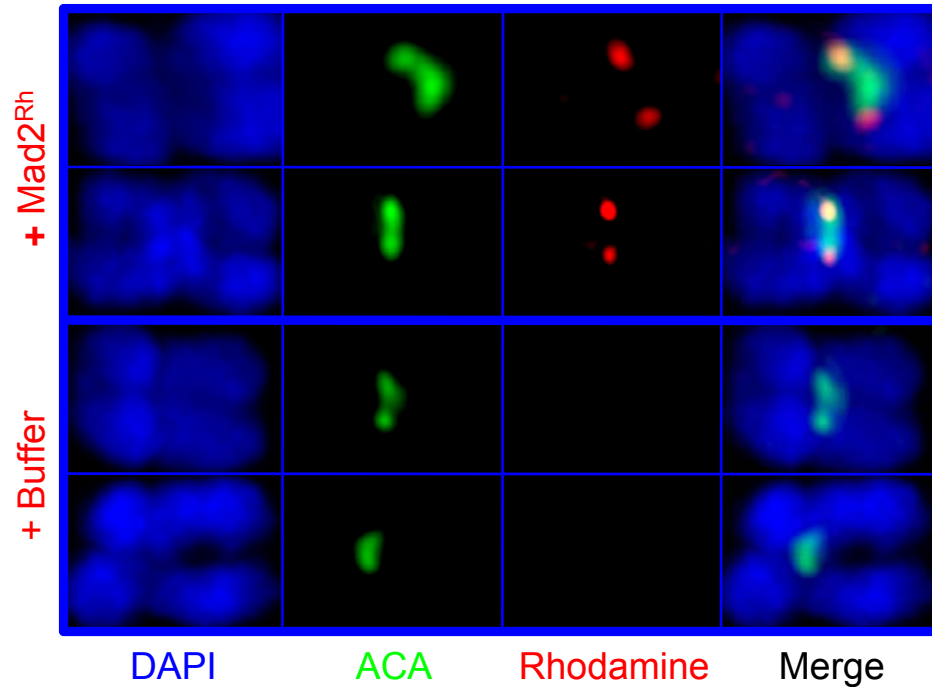
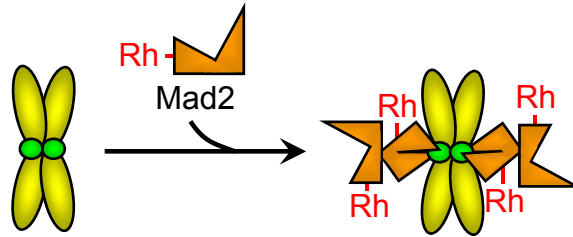
**C**

Figure 10. APC/C ubiquitination assays.

(A) Purified recombinant human Cdh1, Cdc20, Mad2, Bub3 and BubR1, assessed by Coomassie staining. **(B)** Purified recombinant human E1, UbcH10, and N-terminal of cyclin B, assessed by Coomassie staining. **(C)** Immunoprecipitation using immobilized antibodies to the Cdc27 subunit of the interphase *Xenopus* APC/C complex and visualized by silver stain. **(D)** Schematic of APC/C ubiquitination activity assays. Combinations of purified chromosomes and mitotic checkpoint proteins were incubated with Cdc20 prior to addition to APC/C conjugated through Cdc27 antibodies to Affi-prep beads. The beads were subsequently washed to remove unbound proteins and added to an ubiquitination reaction mixture comprised of recombinant E1, UbcH10, myc-cyclin B₁₋₁₀₂, and ubiquitin. Activated APC/C is capable of conjugating ubiquitin chains to its substrates.

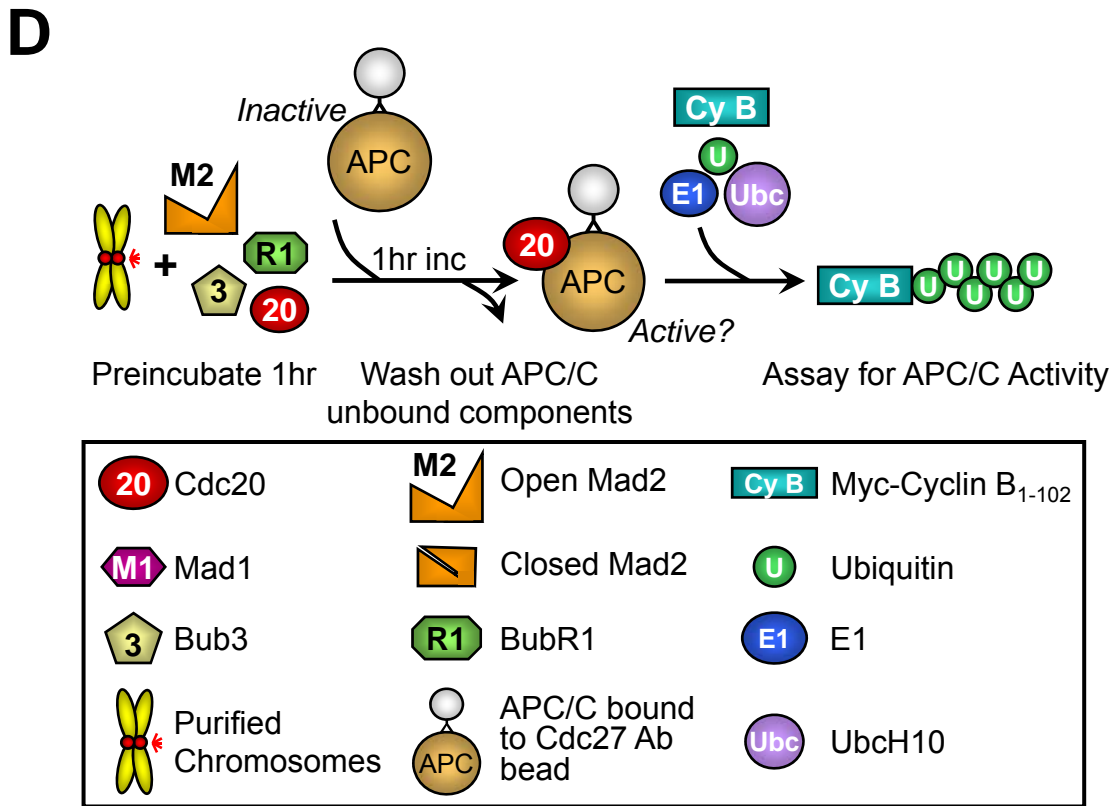
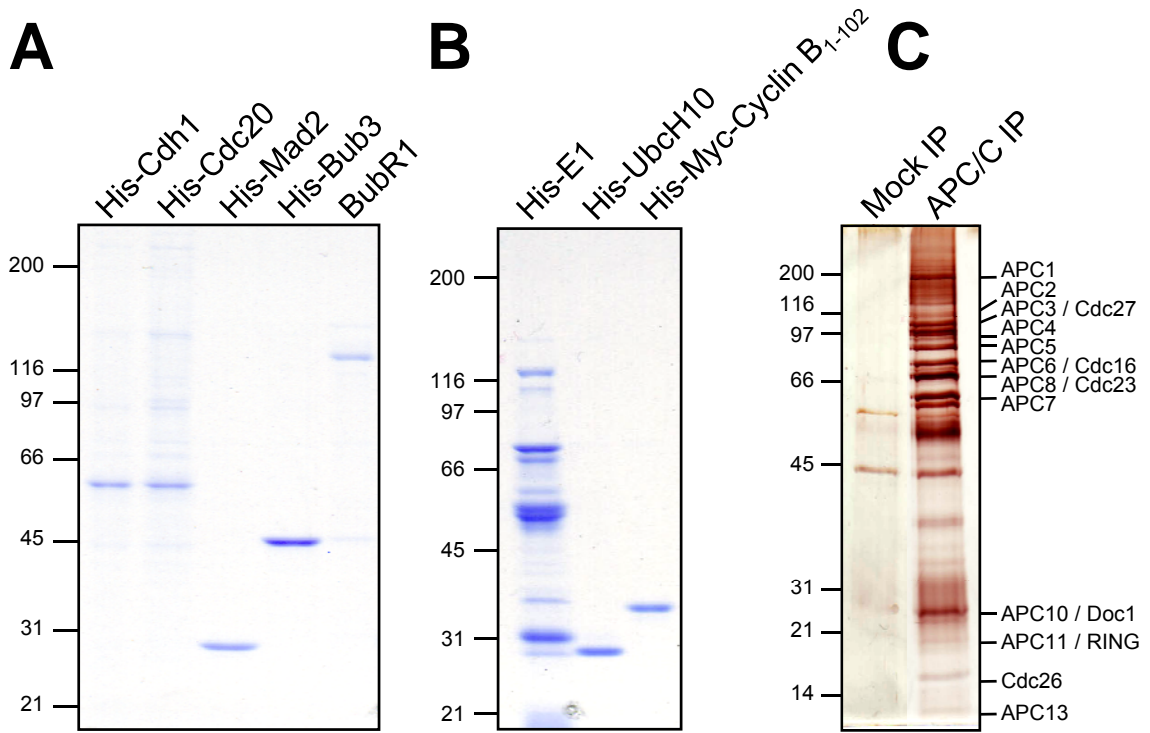


Figure 11. Checkpoint proteins inhibit APC/C activity.

(A) Equal molar amounts of Mad2, Bub3, and BubR1 were incubated with Cdc20 either alone or in various combinations, in the absence of unattached kinetochores. **(B)** APC/C activity was assessed either as the degree of cyclin B ubiquitination (top panel) or as the depletion of the unubiquitinated pool (bottom panel), and **(C)** quantified.

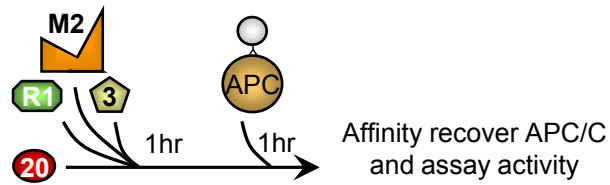
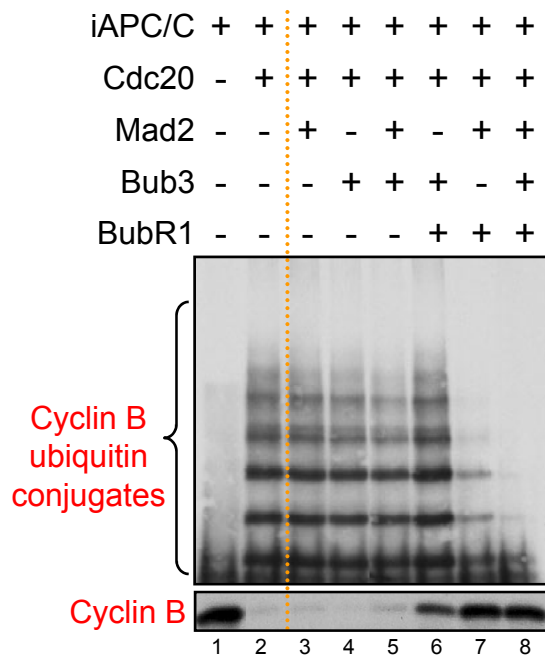
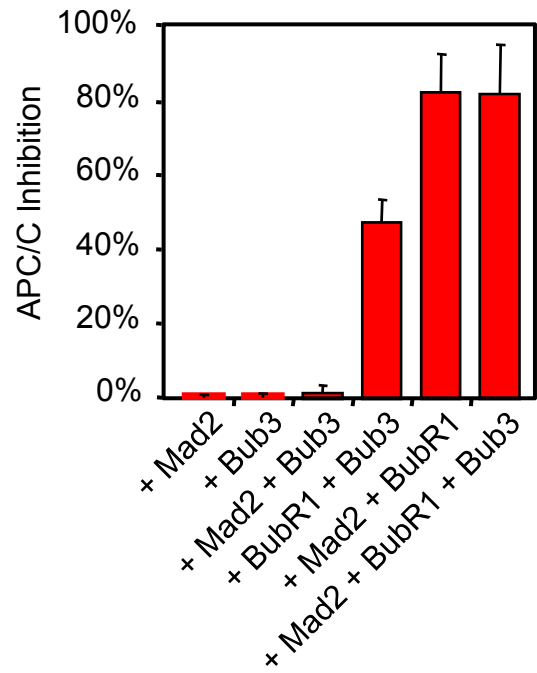
A**B****C**

Figure 12. Synergy of APC/C^{Cdc20} inhibition by addition of Mad2 to Bub3/BubR1.

(A) Synergy of inhibition of APC/C^{Cdc20} from addition of Mad2 to Bub3/BubR1 and Cdc20 prior to APC/C addition and activity measurement compared to corresponding APC/C activity without Mad2 addition. APC/C activity was measured either by **(B)** the presence of ubiquitin-conjugated cyclin B bands (top panel), or by the depletion of the unubiquitinated cyclin B pool (bottom panel), **(C)** the quantification of which is depicted below.

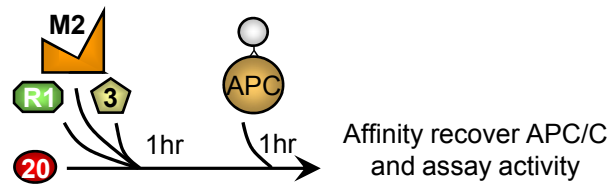
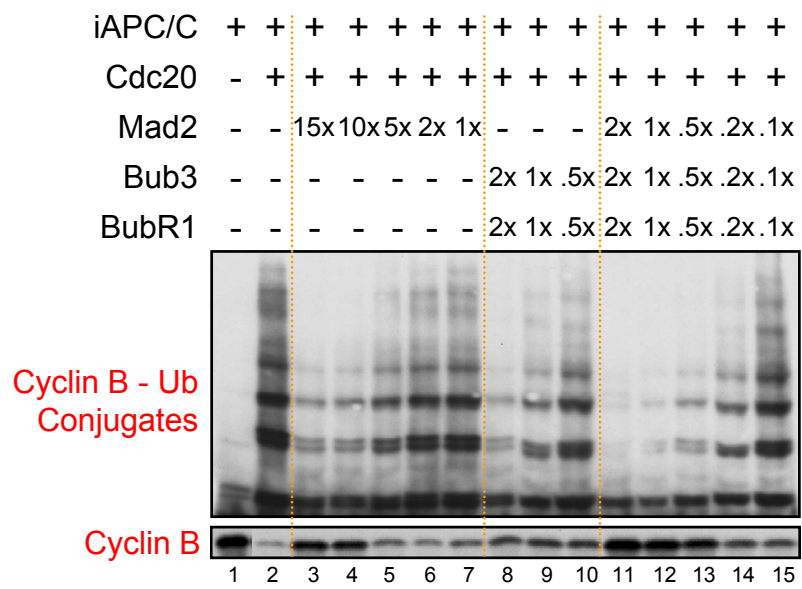
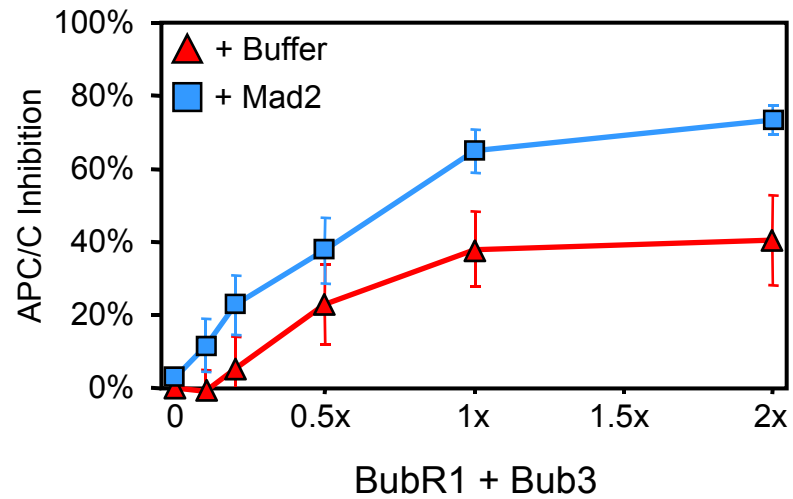
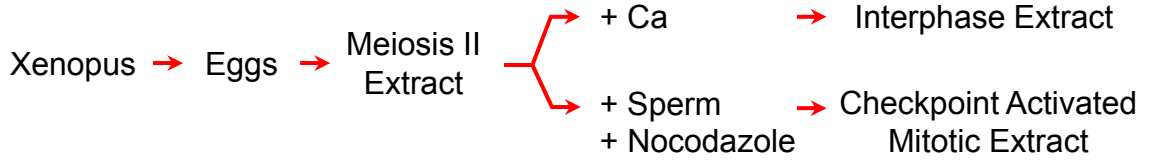
A**B****C**

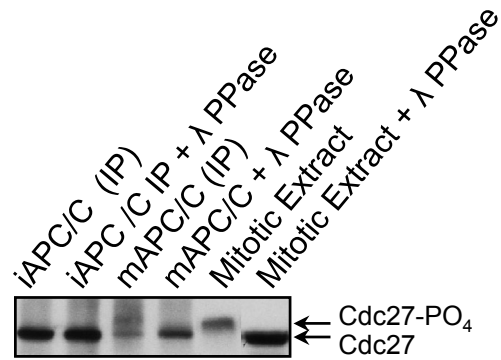
Figure 13. Kinetochores on purified chromosome amplify inhibition of mitotic APC/C^{Cdc20}.

(A) Schematic of *Xenopus* extract preparation for isolation of mitotic APC/C by immunoprecipitation. **(B)** Immunoprecipitated mitotic APC/C is hyperphosphorylated in comparison to interphase APC/C, as judged by immunoblotting for the Cdc27 subunit. Hyperphosphorylation is lost upon phosphatase treatment. **(C)** Mitotic APC/C activity was assessed after addition of increasing amounts of BubR1, Bub3, and Mad2 to Cdc20, either in the presence or absence of chromosomes, and compared to interphase APC/C activity after addition of equivalent amounts of inhibitors. APC/C activity was quantified by the presence of lower mobility ubiquitin-conjugated cyclin B species.

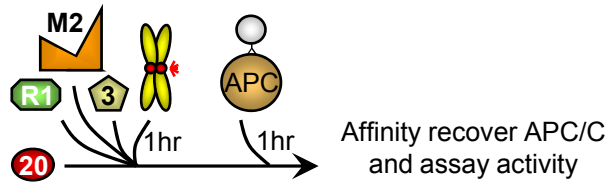
A



B



C



iAPC/C	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-		
mAPC/C	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+		
Cdc20	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
Chromosomes	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+		
BubR1 + Bub3 + Mad2	-	-	1x	.5x	.2x	.1x	-	1x	.5x	.2x	.1x	-	-	1x	.5x	.2x	.1x	-	1x	.5x	.2x	.1x

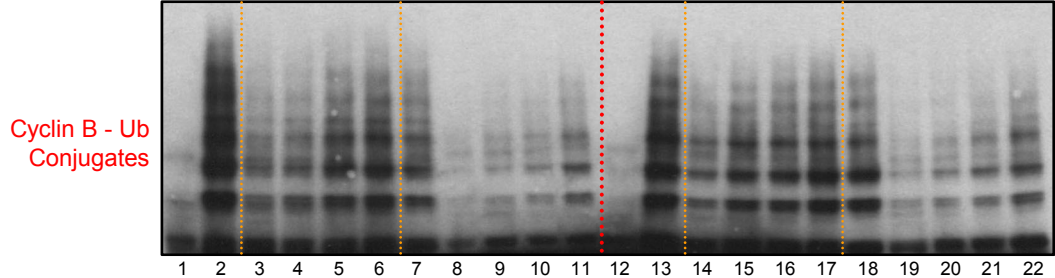


Figure 14. Kinetochore and checkpoint dependent inhibition of APC/C is specific to Cdc20 activation.

(A) Specificity of inhibition by Mad2, BubR1 and Bub3 for blocking activation of APC/C^{Cdc20} was tested by the comparison of Cdc20 versus Cdh1 activated APC/C. APC/C activity was measured either by the presence of ubiquitin-conjugated cyclin B bands (top panel), or by the depletion of the unubiquitinated cyclin B pool (bottom panel), **(B)** the quantification of which is depicted on the right. **(C/D)** BubR1, Bub3, Mad2 and chromosomes were incubated with either Cdc20 or Cdh1 activators, prior to APC/C activity determination. Incubations with Cdc20 rendered the APC/C almost fully inactive, while Cdh1 incubations had no effect on the activity of APC/C.

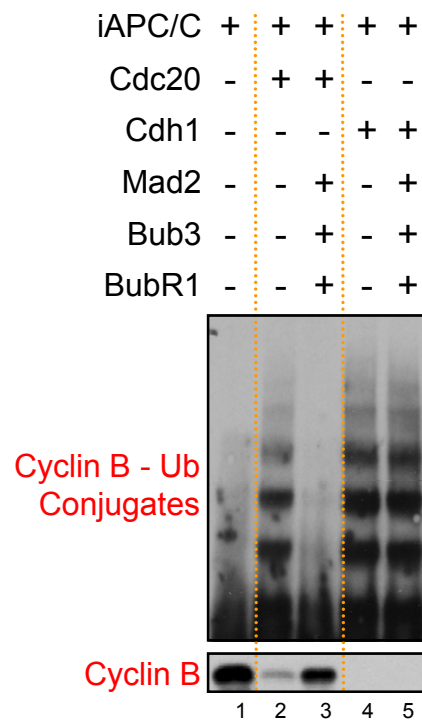
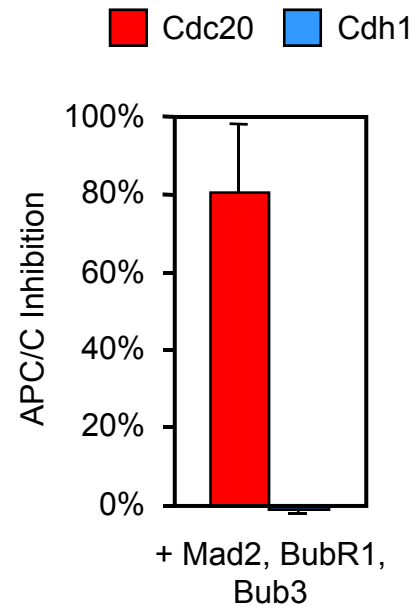
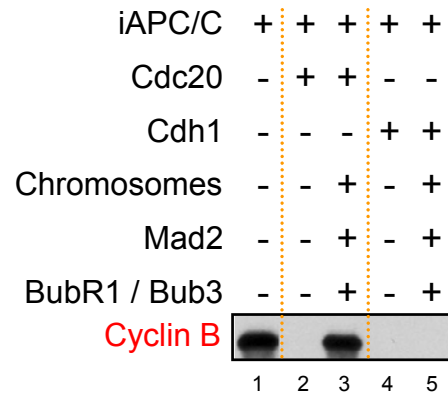
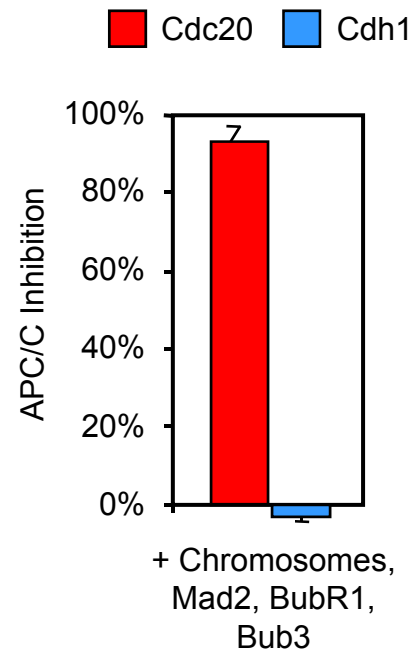
A**B****C****D**

Figure 15. Kinetochores amplify production of an APC/C^{Cdc20} inhibitor.

(A-C) Chromosomes (blue squares) at a final concentration equaling ten unattached kinetochores per cell volume or just buffer (red triangles) were added to increasing concentrations of **(A)** Mad2, **(B)** Bub3/BubR1, or **(C)** both, incubated for 1 hr prior to addition of APC/C, and then assayed for APC/C ubiquitination of myc-cyclin B₁₋₁₀₂. APC/C activity was quantified by the intensity of remaining unubiquitinated cyclin B. Mad2 inhibition of APC/C in the presence of chromosomes increased, while BubR1 inhibition remained unchanged. Chromosomes further amplified APC/C inhibition when added to the combination of Mad2, BubR1, and Bub3 at physiological concentrations.

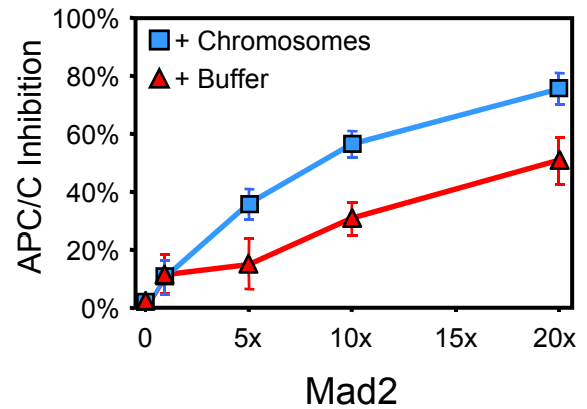
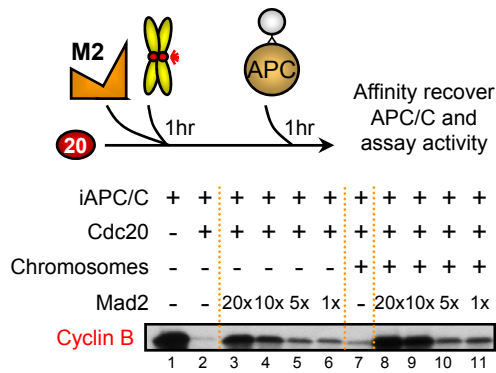
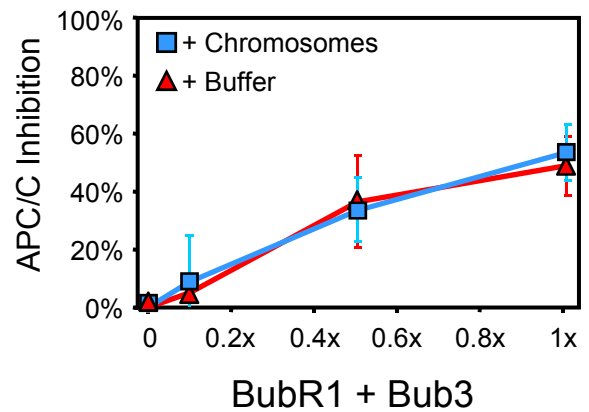
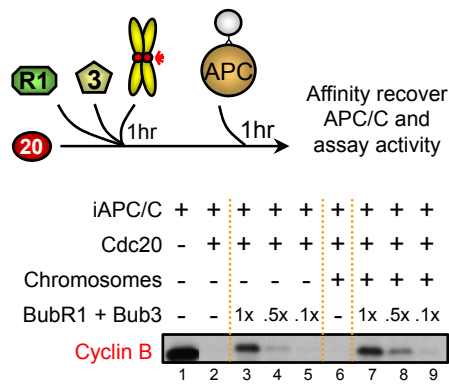
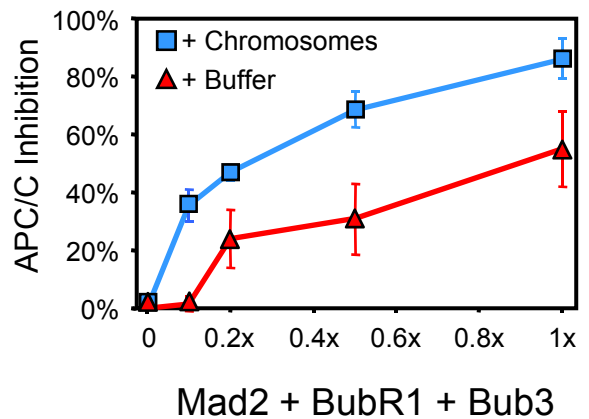
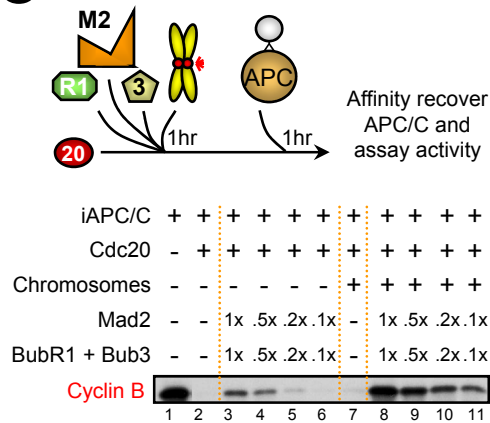
A**B****C**

Figure 16. BubR1 phosphorylation is not stimulated by purified chromosomes.

Purified recombinant BubR1 is phosphorylated at the mitosis specific phospho-residue S670, a phosphorylation which can be removed by phosphatase treatment. Incubation of purified chromosomes with BubR1 did not increase the degree to which BubR1 is phosphorylated at this site.

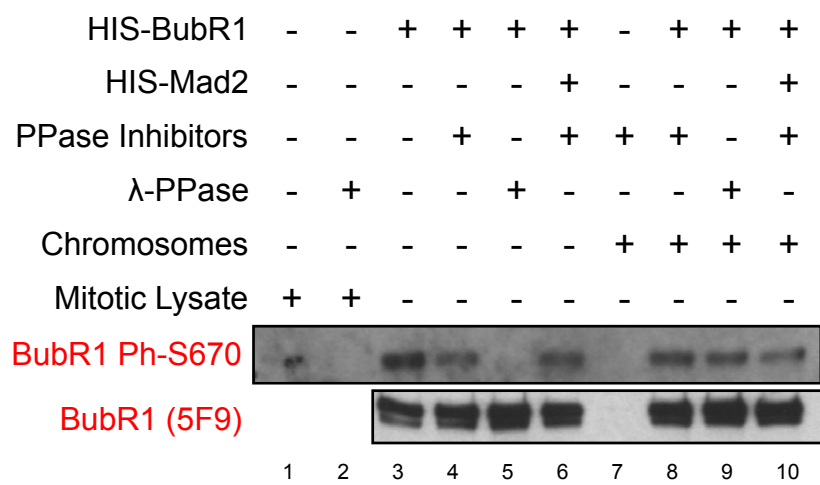
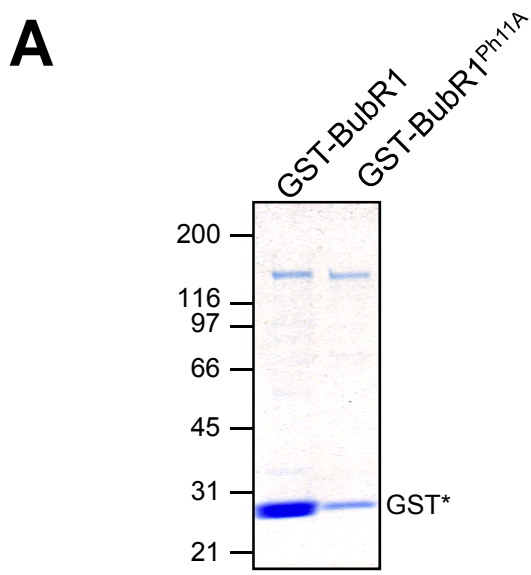


Figure 17. BubR1 phosphorylation does not increase potency as an APC/C inhibitor.

(A) Purified GST-tagged BubR1^{Ph11A}, as assessed by Coomassie stain, **(B)** is mutated at eleven mitosis specific phosphorylation sites. **(C)** Addition of increasing concentration of either (red triangles) wild type BubR1 or (blue squares) BubR1^{Ph11A} to Cdc20 inhibits the ubiquitination of cyclin B. **(D)** Addition of (blue squares) chromosomes to increasing concentrations of BubR1^{Ph11A} compared to (red triangles) BubR1^{Ph11A} alone does not improve inhibition of APC/C.



B

BubR1 ^{Ph11A} Mutated sites:	
S16	S670
S435	S676
S543	S720
S574	S884
S665	T1042
S1043	

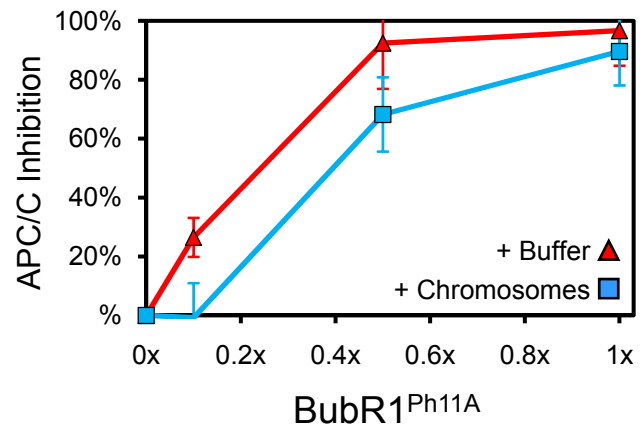
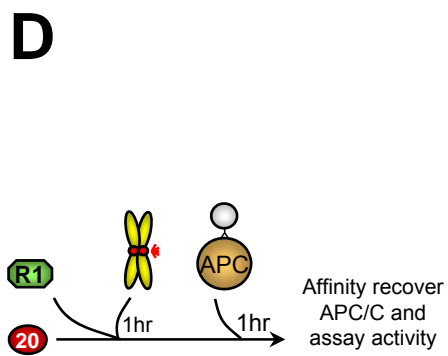
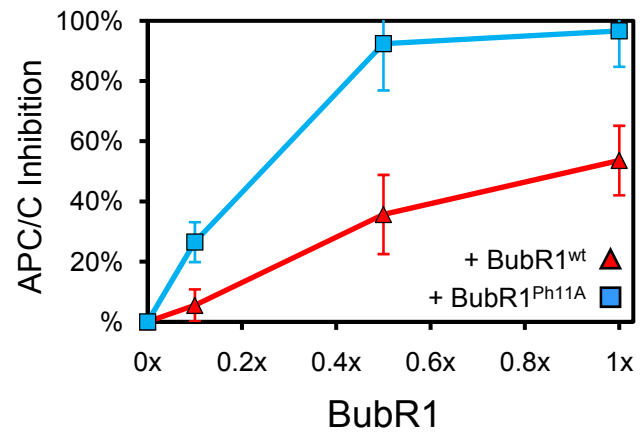
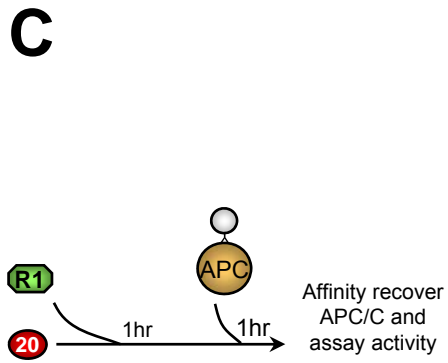


Figure 18. BubR1 kinase activation by ATP addition.

(A) Addition of ATP (blue squares) to increasing concentrations of BubR1 alone (red triangles) did not change its inhibitory potential towards APC/C. **(B)** Incubation of BubR1 with ATP (blue squares) in the presence of chromosomes may increase BubR1 inhibition of APC/C^{Cdc20} at low concentrations of BubR1.

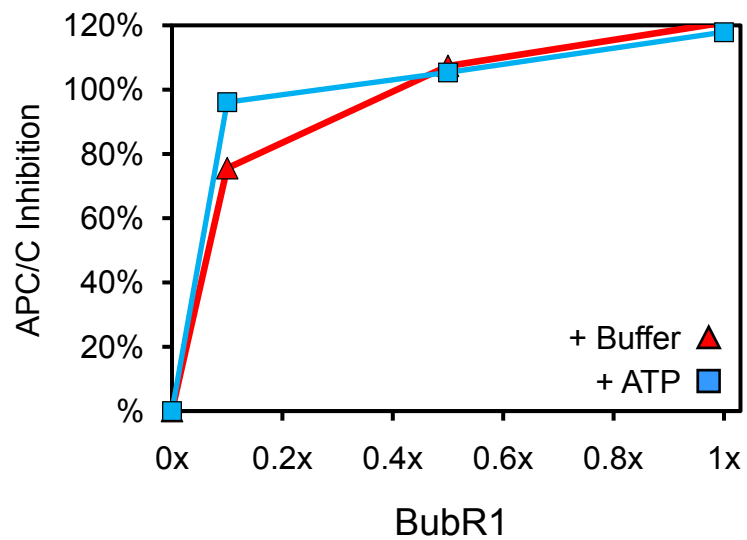
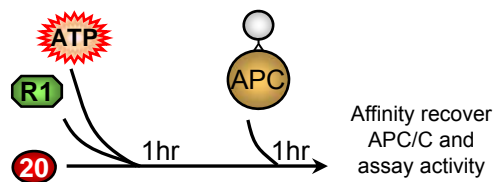
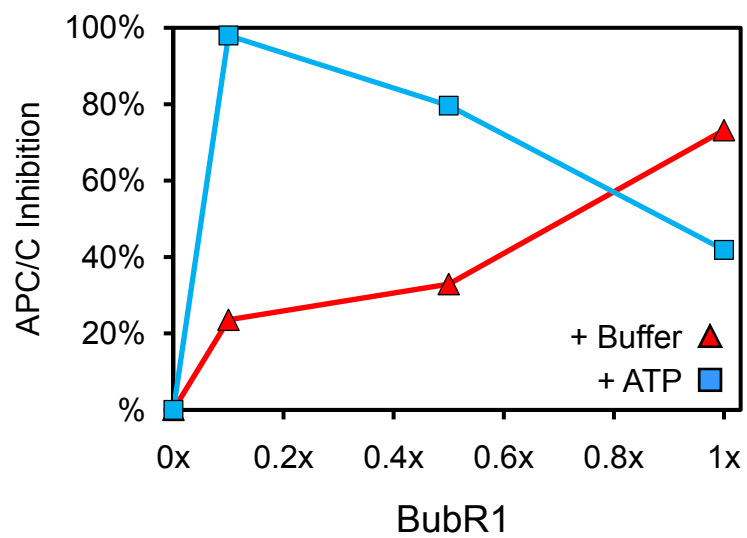
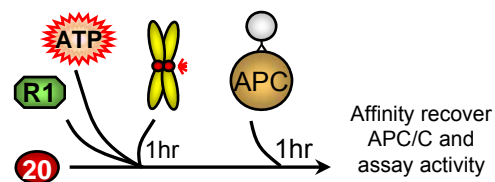
A**B**

Figure 19. BubR1 Kinase activity is not required for the inhibition of APC/C^{Cdc20}.

(A) Purification of kinase inactive BubR1, as determined by Coomassie staining. **(B)** Replacing of (red triangles) wild type BubR1 with (blue squares) BubR1^{K795R} slightly reduced inhibition of APC/C^{Cdc20}. **(C)** Addition of (blue squares) ATP to (red triangles) BubR1^{K795R} did not alter APC/C inhibition.

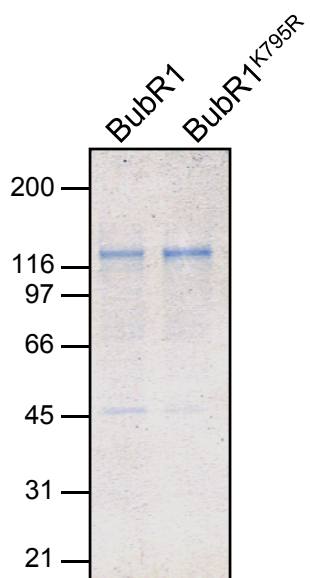
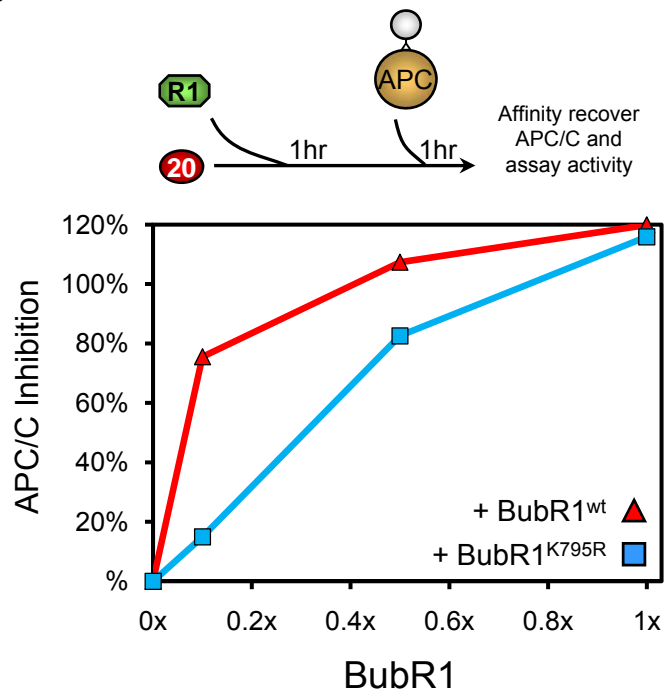
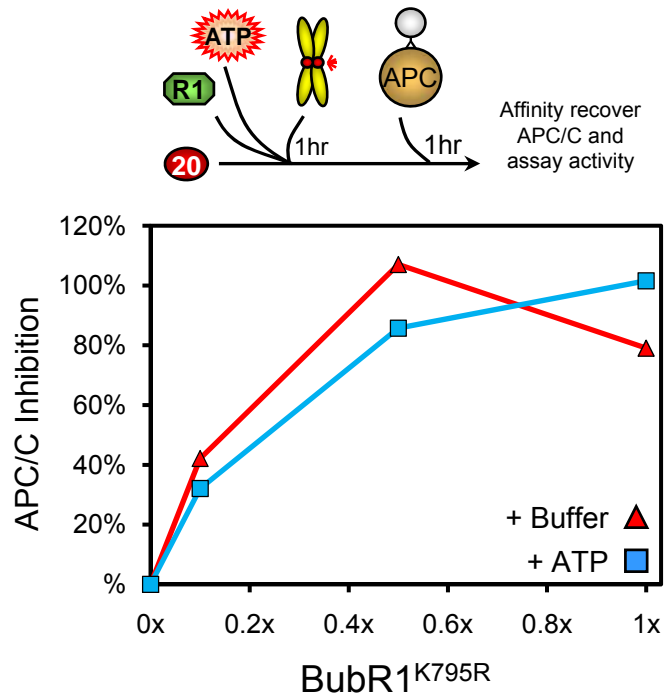
A**B****C**

Figure 20. Kinetochores act catalytically to amplify checkpoint inhibition.

(A) Chromosomes were incubated with Mad2, BubR1, Bub3 and Cdc20. At the indicated time points, (blue squares) a fraction of the incubation was removed, **(B)** assayed for APC/C activity, and **(C)** quantified. Chromosome-mediated catalysis was compared to (red triangles) chromosome-independent inhibitor production over time.

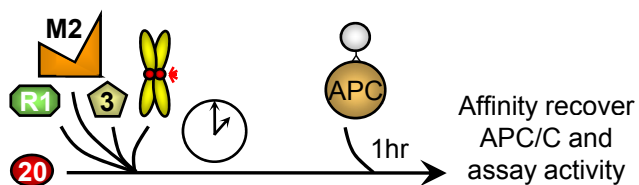
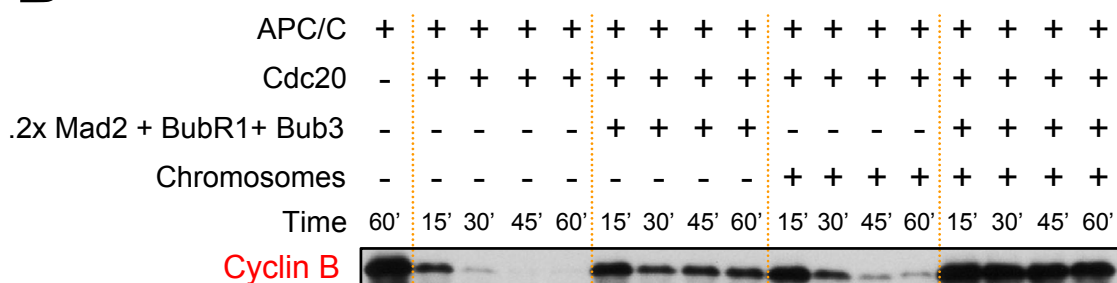
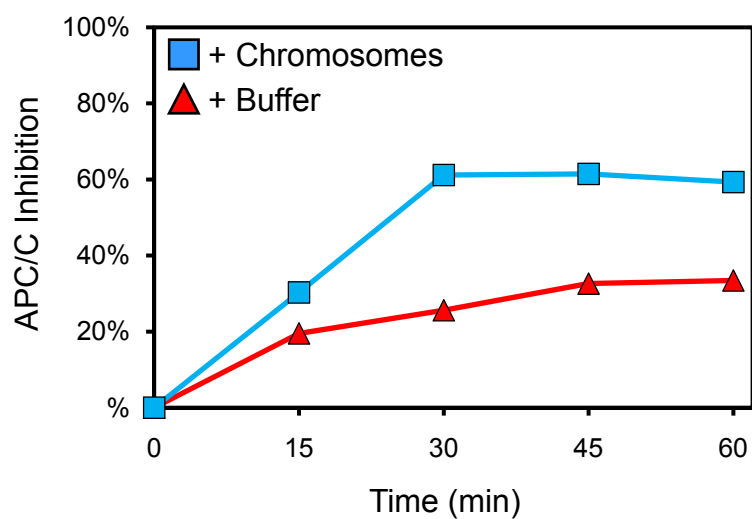
A**B****C**

Figure 21. Mad2 activation by unattached kinetochores is catalytic.

(A) Cdc20 was incubated with a 5-fold excess Mad2 and chromosomes. At the indicated time points, (blue squares) a fraction of the incubation was removed, **(B)** assayed for APC/C activity, and **(C)** quantified. Chromosome-mediated catalysis was compared to (red triangles) chromosome-independent inhibitor production over time.

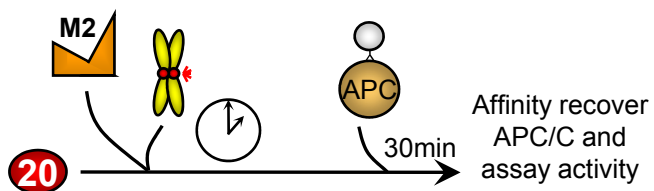
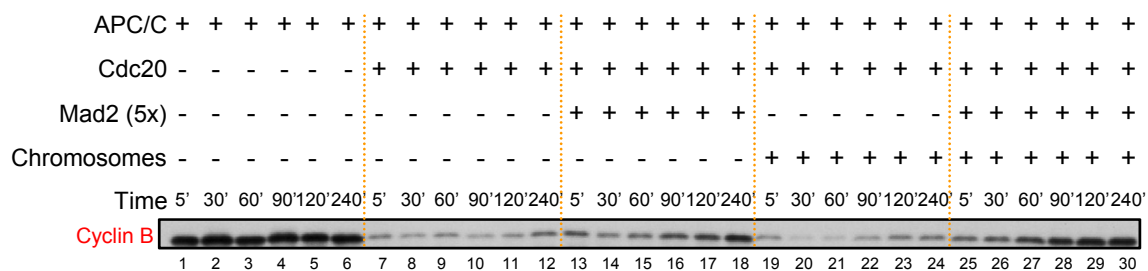
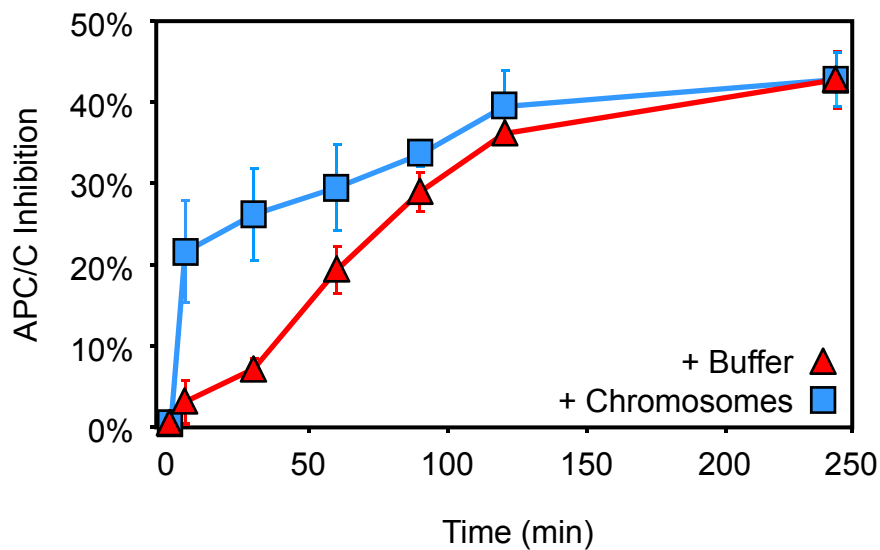
A**B****C**

Figure 22. Mad2 mutant purification and properties.

(A) Table of Mad2 mutant properties (De Antoni et al., 2005; Fang et al., 1998a; Luo et al., 2000; Luo et al., 2004; Sironi et al., 2002), **(B)** depicted for their proposed function. **(C)** Purified recombinant human Mad2^{ΔC}, Mad2^{RQ}, and Mad2^{RQ-ΔC} visualized by Coomassie staining.

A

Name	Mutation(s)	Conformation	Mad1 Binding	Cdc20 Binding	Mad2 Dimerization
wt	---	O/C	+	+	+
Δ C	Δ 196-205	O	-	-	+
RQ	R133E, Q134A	O/C	+	+	-
RQ- Δ C	R133E, Q134A, Δ 196-205	O	-	-	-

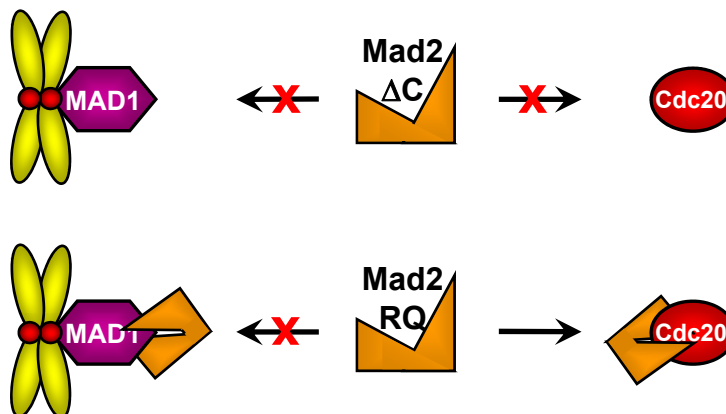
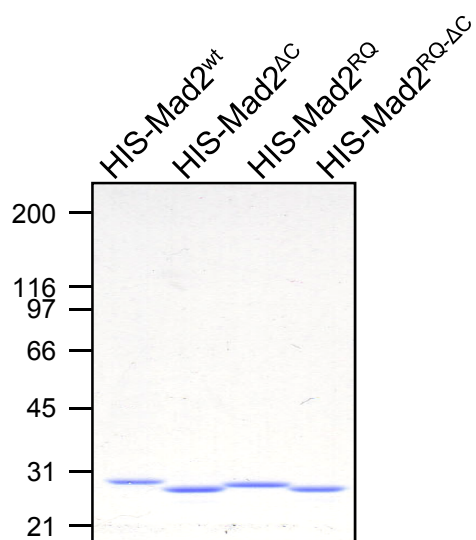
B**C**

Figure 23. Mad2 mutants localize to unattached kinetochores as predicted by the Template Model.

(A) Mad1 binding deficient Mad2^{ΔC} and dimerization incompetent Mad2^{RQ} after purification and covalent conjugation to rhodamine visualized after Coomassie staining. **(B-D)** Purified chromosomes were incubated with rhodamine-labeled Mad2^{wt}, Mad2^{ΔC}, or Mad2^{RQ}, fixed, stained for (blue) DAPI and (green) ACA, and imaged by deconvolution microscopy. **(C)** Chromosomes incubated with rhodamine-labeled Mad2^{wt}, Mad2^{ΔC}, or Mad2^{RQ} were scored for Mad2 kinetochore localization. **(D)** The intensity of Mad2 kinetochore localization was quantified: Mad2^{wt} bound with twice the intensity of Mad2^{RQ}, while Mad2^{ΔC} binding was near background levels.

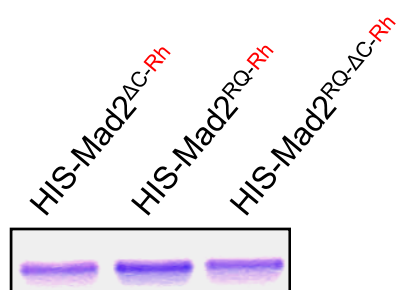
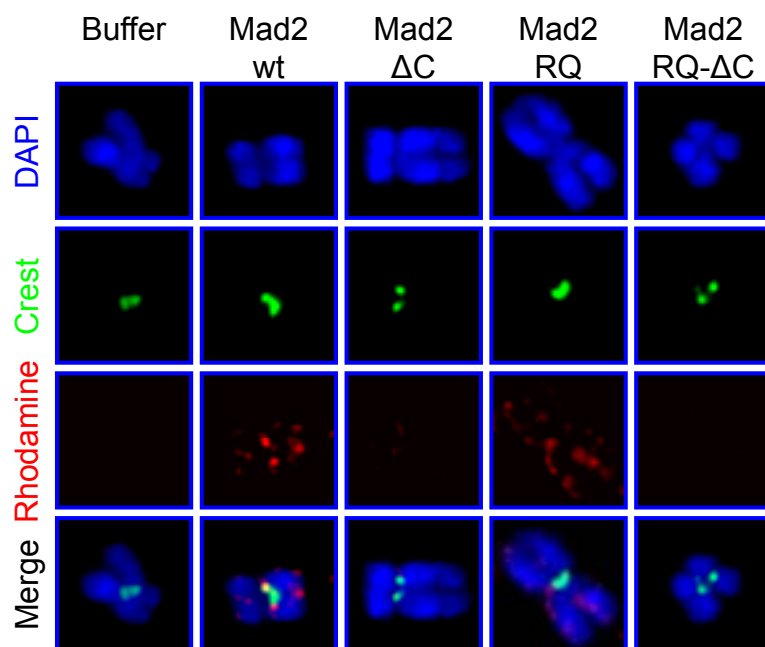
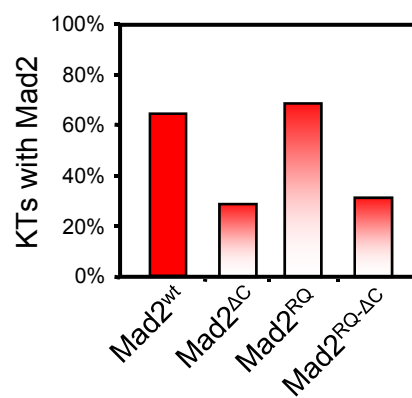
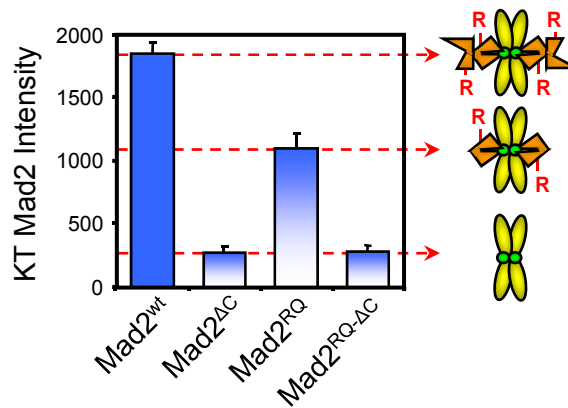
A**B****C****D**

Figure 24. Dimerization incompetent Mad2 can inhibit APC/C in the absence of unattached kinetochores.

(A) Increasing quantities of (red triangles) Mad2^{wt}, (blue squares) Mad2^{ΔC}, (green circles) Mad2^{RQ}, or (yellow diamonds) Mad2^{ΔC-RQ} were incubated for 1 hour with Cdc20 prior to APC/C addition and **(B)** assayed for APC/C ubiquitination of myc-cyclin B₁₋₁₀₂. **(C)** The results were quantified.

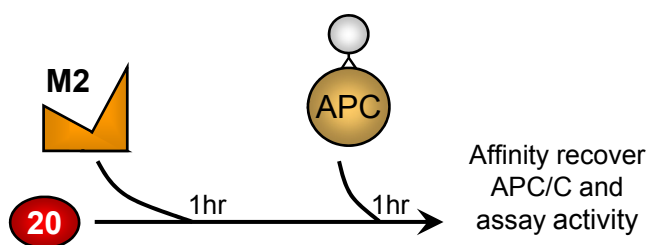
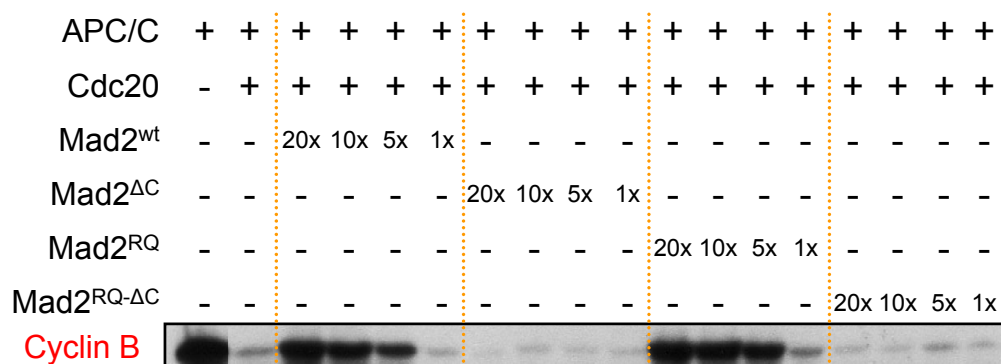
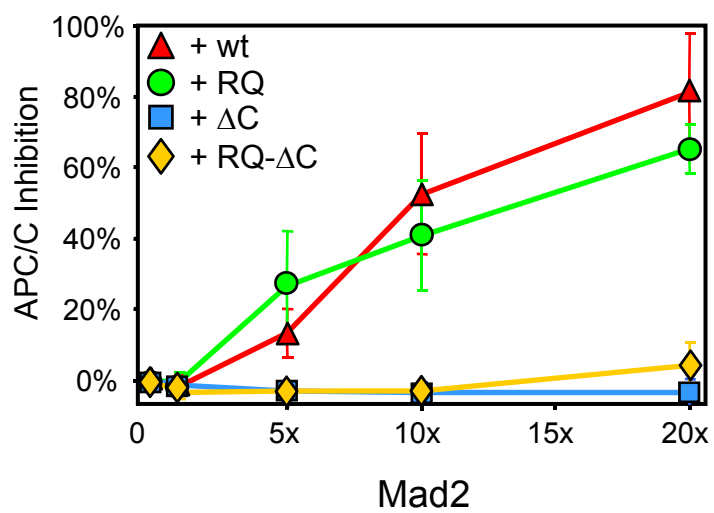
A**B****C**

Figure 25. Kinetochores cannot amplify Mad2^{RQ} mediated inhibition of APC/C^{Cdc20}.

(A) Mad2^{RQ} inhibition of Cdc20 activation of APC/C was assessed by incubating increasing quantities with Cdc20 either in the (blue squares) presence or (red triangles) absence of chromosomes before **(B)** assaying cyclin B ubiquitination, and **(C)** quantifying results. Dimerization deficient Mad2^{RQ} inhibited APC/C at high concentration, but this was not amplified in the presence of chromosomes.

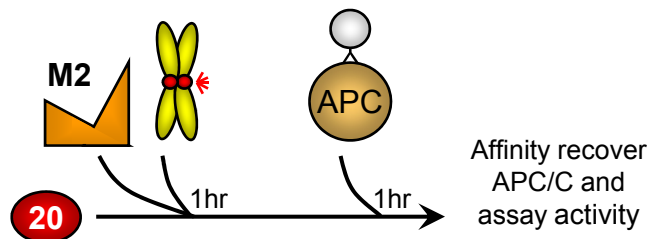
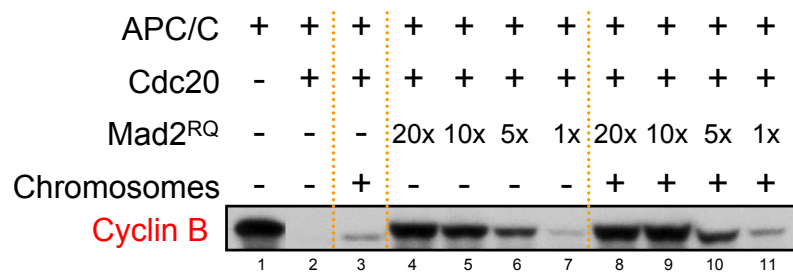
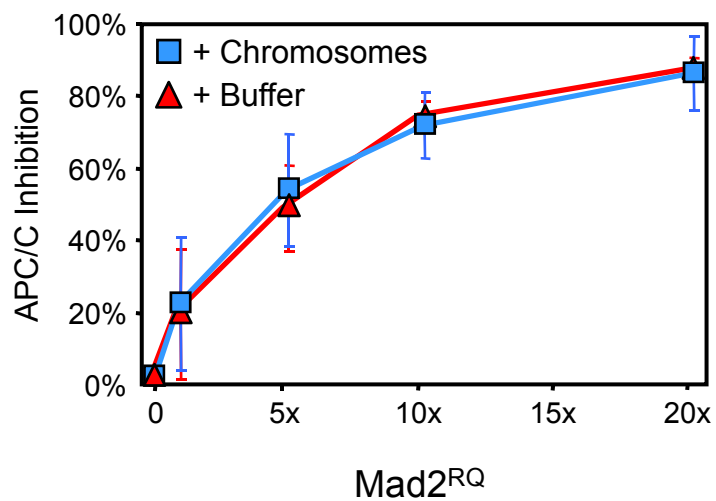
A**B****C**

Figure 26. Mad2 dimerization is required for maximal kinetochore-mediated amplification of a Cdc20 inhibitor by BubR1.

(A) Chromosome amplification of a Cdc20 inhibitor was evaluated with increasing concentrations of (green circles) Mad2^{RQ} or (blue squares) Mad2^{wt}, along with BubR1, Bub3, and Cdc20, either in the presence or (red triangles) absence of chromosomes. **(B)** Inhibition of APC/C^{Cdc20} was assessed by ubiquitination assays and **(C)** quantified.

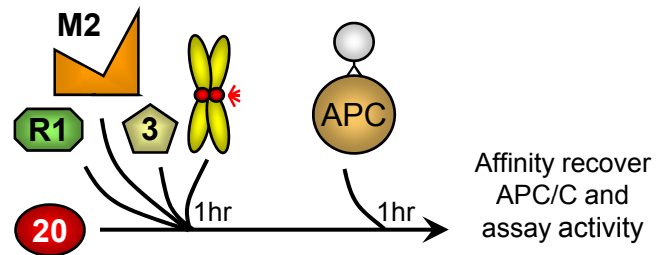
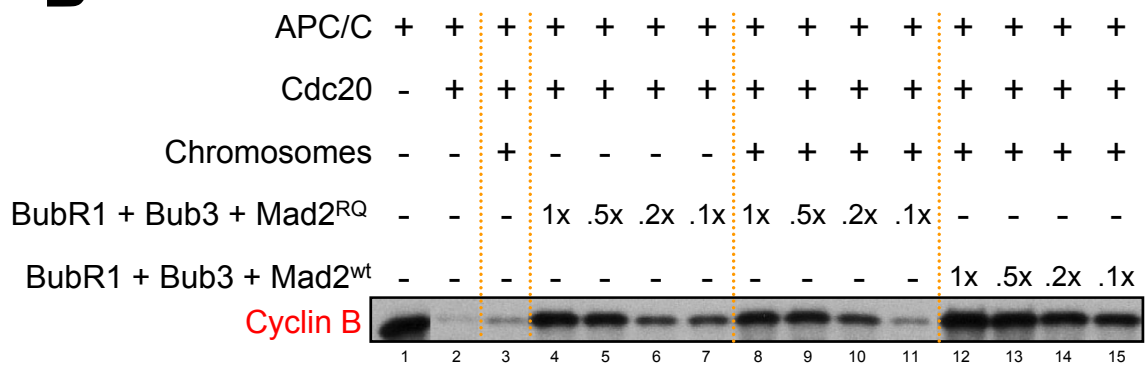
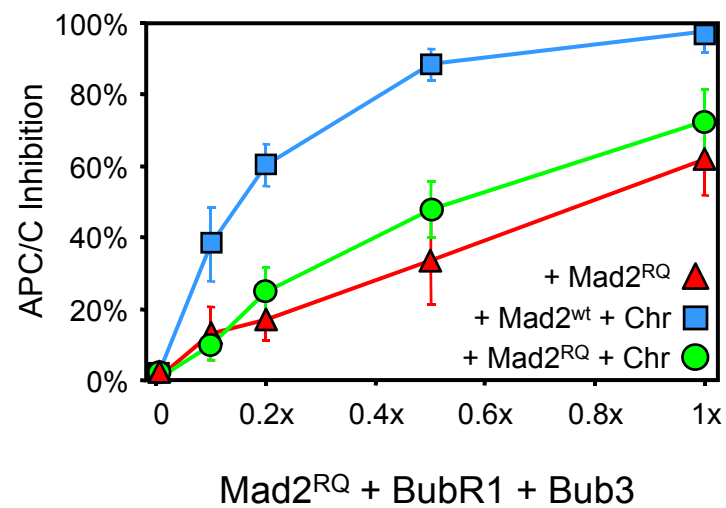
A**B****C**

Figure 27. Mad1 is required for the efficient localization of Mad2 to unattached kinetochores.

(A) Purified Chromosomes were incubated briefly (10 min) with anti-Mad1 antibody, then with rhodamine-labeled Mad2, and finally fixed, stained, imaged by deconvolution microscopy, and **(B)** scored for Mad2 localization.

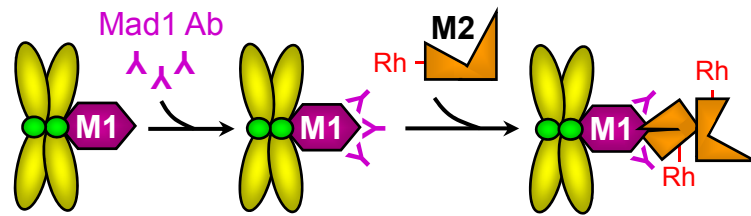
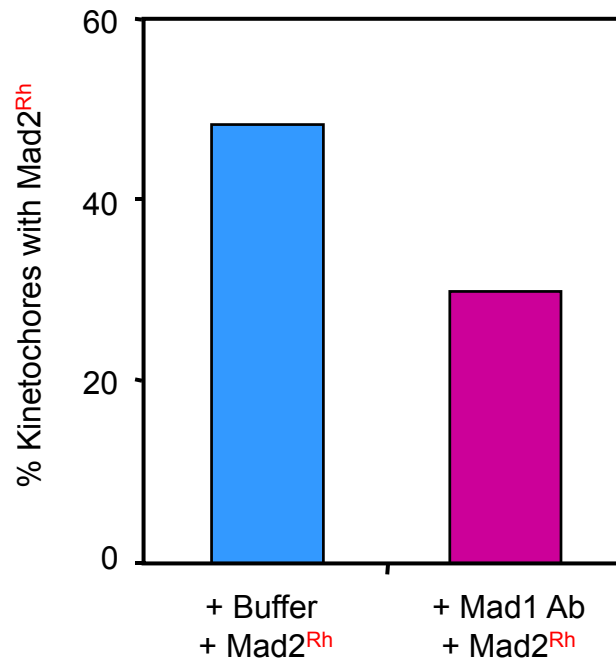
A**B**

Figure 28. Mad1 dependent activation of Mad2 is required for kinetochore-mediated amplification of a Cdc20 inhibitor.

(A) Purified chromosomes were incubated with Mad1 antibody for 1 hour prior to addition of a five-fold excess of Mad2 to Cdc20, and finally **(B)** addition to APC/C ubiquitination assays. **(C)** The resulting inhibition of AP/CC^{Cdc20} was measured. Chromosomes enhanced Mad2 inhibition of APC/C, but this was blocked by treatment with the Mad1 antibody.

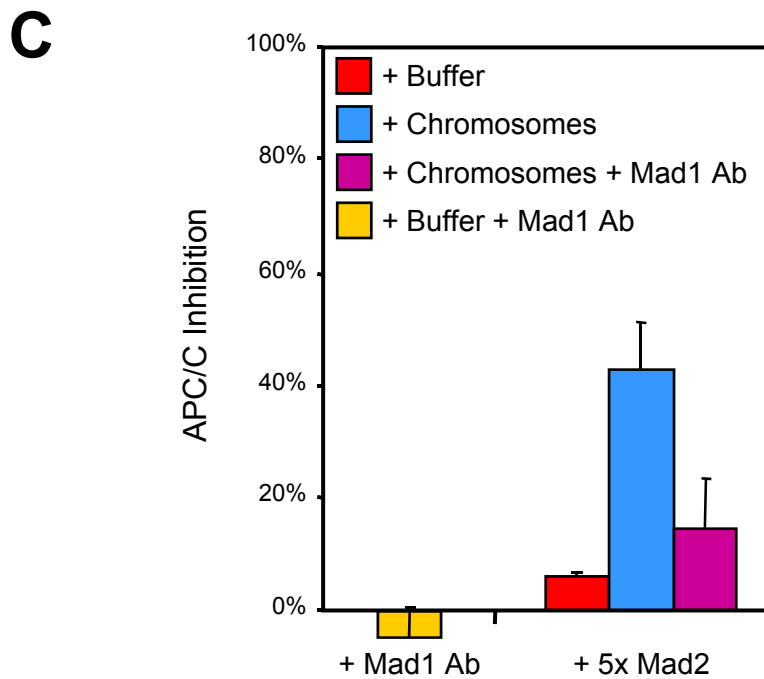
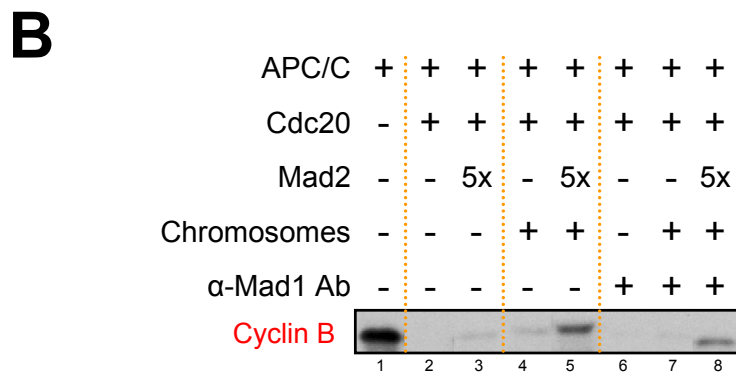
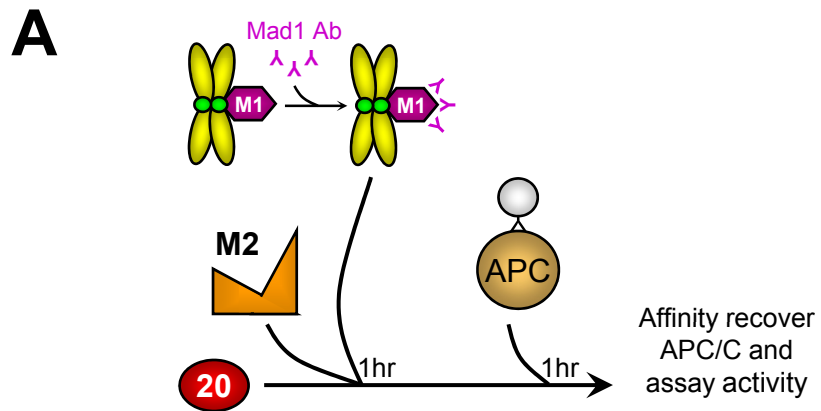
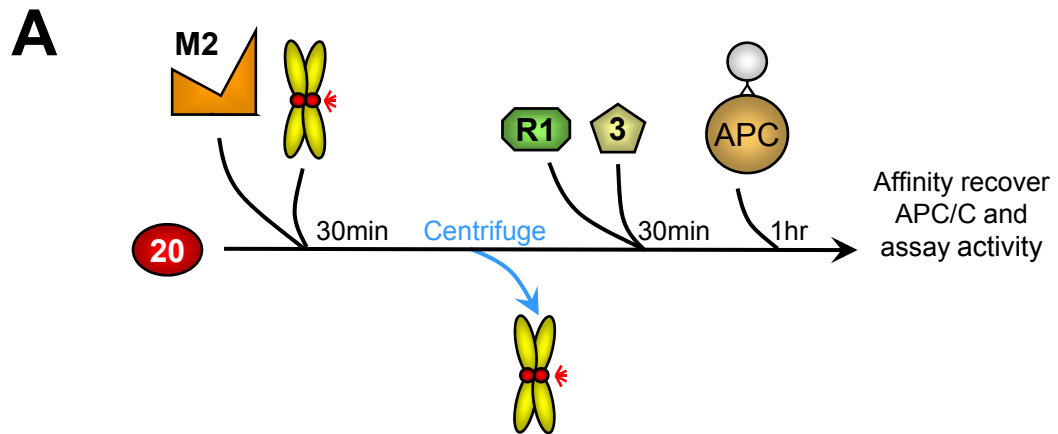


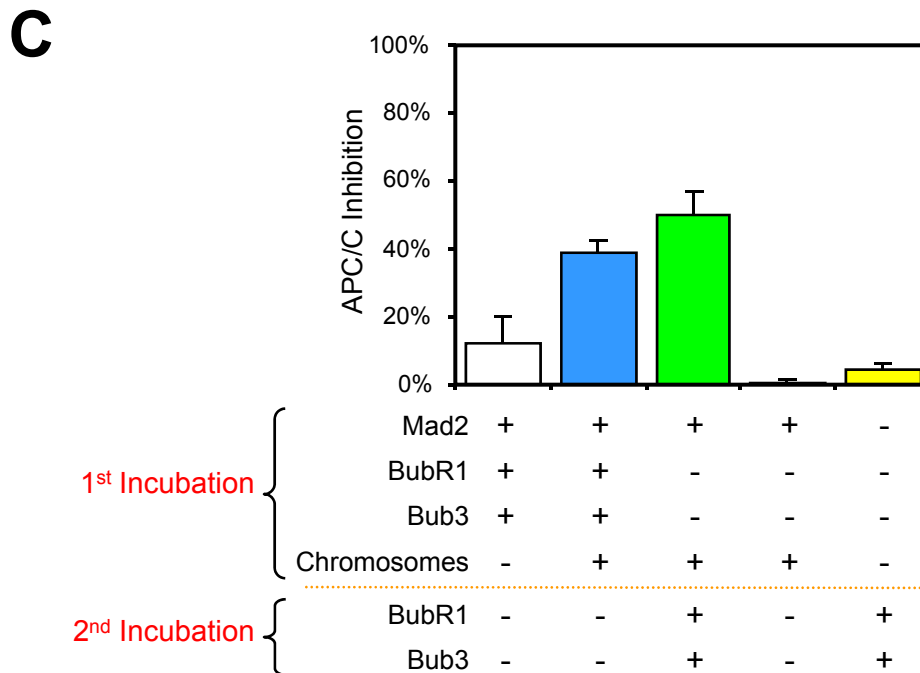
Figure 29. Production by unattached kinetochores of a Cdc20 inhibitor through sequential involvement of Mad2 and BubR1.

(A) Chromosomes were initially incubated with Mad2 and Cdc20 (1:5); the chromosomes were subsequently removed, and BubR1/Bub3 added to the supernatant fraction after chromosome removal (green). **(B)** The resulting ubiquitination activity was **(C)** quantified and compared to (blue) inhibition produced by incubating chromosomes with Mad2, BubR1, and Bub3 prior to chromosome removal or (white) no chromosomes added.



B

APC/C	+	+	+	+	+	+	+	+
Cdc20	-	+	+	+	+	+	+	+
Mad2 (.2x)	-	-	+	-	+	+	-	+
BubR1 + Bub3 (.2x)	-	-	+	-	-	+	-	-
Chromosomes	-	-	-	+	+	+	-	+
2nd Incubation:								
BubR1 + Bub3 (.2x)	-	-	-	-	-	-	+	+
Cyclin B								
	1	2	3	4	5	6	7	8



CHAPTER 3.

Unattached Kinetochores Promote the Association of BubR1/Bub3 with the APC/C-Cdc20 to Relay a Checkpoint Active State.

Anita Kulukian, Joo Seok Han, Don Cleveland

Abstract:

With the discovery that APC/C is the downstream target of the mitotic checkpoint, there has been much theorizing about how kinetochores propagate and relay a wait anaphase signal to inhibit APC/C activation. Here we demonstrate that preactivated APC/C can still be inhibited by the checkpoint with Cdc20 still bound to it, and thus discount sequestration of Cdc20 as the sole means by which APC/C is inactivated. Unattached kinetochores are demonstrated to produce a soluble Cdc20 inhibitor which cannot differentially inhibit cyclin A ubiquitination. By activating Mad2, purified chromosomes promote the association of BubR1/Bub3 with APC/C^{Cdc20}. These data support a model in which immobilized Mad1/Mad2 at kinetochores provides a template for initial assembly of Mad2 bound to Cdc20 that is then converted to BubR1-Cdc20 as sequentially produced mitotic checkpoint inhibitors.

Introduction:

With the discovery that APC/C is the downstream target of the mitotic checkpoint, there has been much speculation about how kinetochores propagate and relay an APC/C inhibitory signal. Logic has dictated that the wait anaphase signal must be diffusible since a single unattached kinetochore must be able to inhibit the whole of cellular APC/C (Cleveland et al., 2003), which is predominantly localized to centrosomes and the mitotic spindle (Kraft et al., 2003; Topper et al., 2002; Tugendreich et al., 1995). What has been demonstrated is that the signal has limited diffusibility. When two Ptk1 cells undergoing mitosis were fused together, the unattached chromosomes of one spindle were incapable of preventing anaphase onset of the adjacent spindle with aligned chromosomes (Rieder et al., 1997). Yet to date, no additional molecular or cell biological experimental data has been put in press regarding the diffusible nature of the checkpoint. Instead, *in silico* modeling of the checkpoint has confirmed the idea that checkpoint arrest cannot be sustained in the absence of a diffusible inhibitor. There must be a kinetochore-dependent generation of an inhibitor which diffuses away from the kinetochore (Doncic et al., 2005; Sear and Howard, 2006).

With the demonstration of Mad2 and BubR1 binding to Cdc20 (Fang et al., 1998; Tang et al., 2001), several theories have been put forth as to how these factors can mechanistically neutralize Cdc20 stimulation of APC/C

substrate ubiquitination (Figure 31) and how unattached kinetochores might contribute to that inhibition (Figure 32). Because the binding of Mad2 or BubR1 to Cdc20 is stoichiometric (Fang et al., 1998; Tang et al., 2001), initial theories hypothesized that Mad2 and/or BubR1 sequester Cdc20 away from APC/C to preempt their interaction (Figure 31A,B) (Fang et al., 1998; Reimann et al., 2001; Tang et al., 2001). These theories stemmed from the observation that pre-incubation of Cdc20 with BubR1 or Mad2 reduced the amount that immunoprecipitated with the APC/C (Tang et al., 2001). One study found that the binding sites for Mad2 and Cdc27 on Cdc20 were distinct but overlapping, suggesting that the binding of Cdc20 to either Mad2 or to the APC/C was mutually exclusive (Zhang and Lees, 2001). Additionally, Cdc20 can be found in a complex with with Mad2 and BubR1, a portion of which does not associate with the APC/C (Sudakin et al., 2001; Wang et al., 2006). Yet sequestration as a means for inactivating the APC/C is not fully sustained by computation modeling (Ibrahim et al., 2008a; Ibrahim et al., 2008b). Additionally, sequestration does not explain how APC/C that does not interact with Cdc20 can still ubiquitinate and mediate the degradation of cyclin A in prophase (den Elzen and Pines, 2001; Geley et al., 2001). Cyclin A degradation requires active APC/C^{Cdc20} (Geley et al., 2001).

Stoichiometric activation of APC/C by Cdc20 is, in itself, questionable (Figure 30A). APC/C in *Xenopus* extracts which are immunodepleted of Cdc20 can still recognize the D-Box motifs of substrates (Yamano et al., 2004),

leading to the proposal that even transient interaction of Cdc20 with the APC/C is sufficient to activate it (Figure 30B). If the interaction of Cdc20 with APC/C is a weak one with a high dissociation coefficient, then the binding of inhibitory factors should be sufficient to dissociate Cdc20 from APC/C (Figure 31D). However, a different study reported that substrates interact optimally with APC/C when the activator forms a stably associated complex with it (Passmore and Barford, 2005).

Additional evidence has shown that both Cdc20 and its inhibitors interact with the APC/C *in vivo* during checkpoint activation, which in itself calls into question whether Cdc20 is ever sequestered away from the APC/C. Fluorescence Correlation spectroscopy experiments concluded that Cdc20 conformers can be found in a large megadalton complex during interphase, with only a proportion of it dissociating from the APC/C in prometaphase (Wang et al., 2006). Both Mad2 and BubR1 associate with APC/C (Chan et al., 1999; Fang et al., 1998; Kallio et al., 1998; Wu et al., 2000), with the latter mimicking the prometaphase pattern observed for Cdc20-APC/C interaction (Braunstein et al., 2007; Morrow et al., 2005). All of this points towards checkpoint factors directly interacting with Cdc20 that is bound to APC/C to inhibit its activity (Figure 31C), a model which is supported by *in silico* evidence as well (Ibrahim et al., 2008a; Ibrahim et al., 2008b).

In spite of how BubR1 and Mad2 might inhibit APC/C activity, it is still not apparent what role unattached kinetochores play in terms of relaying a

checkpoint active state to shut off APC/C activity (Figure 32). Mitotic checkpoint complexes (containing BubR1/Mad3, Mad2, and Cdc20), may form which bind to APC/C preferentially in mitosis (Sudakin et al., 2001), but MCC-like complexes can also be found in interphase cells (Sudakin et al., 2001) or in the case of yeast, when a functional kinetochore is not available (Fraschini et al., 2001). With the observation that a proportion of APC/C can be found at unattached kinetochores (Acquaviva et al., 2004; Hames et al., 2001; Jorgensen et al., 1998; Kurasawa and Todokoro, 1999; Topper et al., 2002; Vigneron et al., 2004; Zhang and Lees, 2001), it has been speculated that the role of the kinetochore is to sensitize APC/C for inhibition by a pre-made inhibitor (Figure 32B) (Acquaviva et al., 2004; Sudakin et al., 2001). However, computational modeling has predicted that anaphase onset cannot be restrained solely by the recruitment of APC/C to a single kinetochore (Doncic et al., 2005).

In contrast, it has also been proposed that the role of unattached kinetochores is to catalyze the formation of inhibitory complexes (Figure 32D) (Weaver and Cleveland, 2005). Significant evidence exists that Mad1 localized to kinetochores during prometaphase recruits Mad2 to catalyze its conformational change into a structure that can more readily bind to Cdc20 (Mapelli and Musacchio, 2007), a theory that we show to be correct in Chapter 2. However, as also demonstrated, Mad2, while activated by kinetochores, cannot fully restrain APC/C activity on its own, and BubR1 must function

downstream of it to fully restrain anaphase entry. With this in mind, we tested the above proposed models to determine the role of kinetochores in producing an APC/C inhibitor and the mechanism by which that inhibitor restrains APC/C activity.

Results:**A soluble kinetochore-derived Cdc20 inhibitor.**

To test whether a soluble kinetochore derived inhibitor of Cdc20 can be produced, chromosomes with unattached kinetochores were incubated with BubR1, Bub3, Mad2, and Cdc20, but then removed prior to the addition of APC/C (Figure 33). In the absence of chromosomes, incubation of Cdc20 and a low level of BubR1, Bub3, Mad2 and Cdc20, followed by subsequent addition of APC/C, produced almost fully active APC/C^{Cdc20}. Parallel incubation of the same amounts of BubR1, Bub3, Mad2 and Cdc20 but now in the presence of chromosomes, amplified APC/C inhibition greater than threefold, independently of whether chromosomes were removed prior to APC/C addition. Thus, a soluble inhibitor(s) amplified by unattached kinetochores is produced independently of APC/C.

A kinetochore-derived APC/C inhibitor which blocks ubiquitination of cyclin A *in vitro*.

APC/C ubiquitinates cyclin A and Nek2A during prometaphase, at a time when it cannot ubiquitinate cyclin B and securin because of checkpoint arrest (Geley et al., 2001; Hames et al., 2001). To test if the soluble APC/C inhibitor could confer substrate specificity of APC/C during prometaphase, such that it allowed for the ubiquitination of specific substrates but selectively

blocked the ubiquitination of others, cyclin B was substituted by purified cyclin A (Figure 34B) as a substrate of APC/C in ubiquitination assays. While both Cdc20 and Cdh1 could mildly stimulate the ubiquitination of cyclin A substrates (Figure 34C), addition of BubR1, Bub3, and Mad2 to APC/C^{Cdc20} inhibited ubiquitination of cyclin A as well (Figure 34D). Thus, checkpoint inhibitors could not mediate substrate selection for the APC/C.

Kinetochores-enhanced inhibition of Cdc20-bound to APC/C.

Models of APC/C inhibition in mitosis initially focused upon sequestration of Cdc20 (Fang et al., 1998; Reimann et al., 2001; Sudakin et al., 2001; Tang et al., 2001) as the mode of preventing Cdc20 activation of APC/C. An alternative is for inhibition by a checkpoint derived inhibitor binding directly to Cdc20 already bound to APC/C. To distinguish between these models, inhibition of APC/C^{Cdc20} activity was assessed either by pre-incubation of chromosomes, Bub3/BubR1, Mad2, and Cdc20 followed by addition of APC/C (Figure 35A) or by addition of chromosomes, Bub3/BubR1, and Mad2 to Cdc20 pre-bound to APC/C (Figure 35B). At all concentrations of checkpoint components, inhibition of APC/C^{Cdc20}-mediated ubiquitination of cyclin B₁₋₁₀₂ was comparable following co-incubation with Cdc20 or after pre-activation by binding of Cdc20 to APC/C (Figure 35C,D). Thus, APC/C^{Cdc20} can be directly inhibited by a kinetochore derived inhibitor(s) and a model of simple sequestration of Cdc20 cannot be the sole means by which APC/C is

held inactive for cyclin B ubiquitination.

Kinetochores facilitate Bub3/BubR1 binding to APC/C^{Cdc20}.

Pull down assays in which co-incubated GST tagged Cdc20 and BubR1 were eluted from beads by glutathione addition demonstrated that BubR1 could bind to Cdc20 in the absence of Mad2 or chromosomes (Figure 36). However, Cdc20 that is unbound to APC/C is irrelevant to checkpoint signaling because it is APC/C^{Cdc20} activity which is regulated in prometaphase. To probe whether the unattached kinetochores on purified chromosomes altered the composition of proteins bound to APC/C, a peptide-derived antibody to Cdc27 (Herzog and Peters, 2005) was used to affinity purify APC/C. After preincubation of Cdc20, BubR1, Bub3, Mad2 and chromosomes, bead-bound APC/C was added and incubated for one hour. The APC/C beads were recovered, and APC/C and proteins bound to it were released by addition of a competing Cdc27 peptide. Immunoblotting was used to determine which protein components remained bound to the APC/C complex (Figure 37).

Similar amounts of Cdc20 co-immunoprecipitated with APC/C regardless of the concentrations of co-incubated checkpoint components (Figure 37B). While a proportion of Mad2 has previously been reported to be associated with APC/C and such binding was proposed to be mediated through Cdc20 (Fang et al., 1998; Kallio et al., 1998), Mad2 binding to APC/C was not Cdc20 dependent (Lane 11). Small, but variable, proportions of Mad2

and dimerization incompetent Mad2^{RQ} bound to APC/C independent of chromosomes, suggesting a direct affinity of Mad2 for APC/C that was modestly increased by the presence of chromosomes (Figure 37,38B,D). Neither BubR1 nor Bub3 bound APC/C in the absence of Mad2. Bub3/BubR1 did, however, bind to APC/C in a Mad2 dependent manner that at all concentrations tested was further enhanced by up to 4 fold by chromosomes (Figure 37, compare lanes 4 with 9 and 10; Figure 38B,C,D). However, the number of BubR1 molecules bound to APC/C^{Cdc20} after incubation with chromosomes was always greater than the number of bound Mad2 molecules, as seen by a stoichiometry of greater than 1:1 (Figure 39), inconsistent with MCC-like complexes which predict equal stoichiometries of Mad2 and BubR1/Bub3. This chromosome-enhanced BubR1/Bub3 binding was not supported by the dimerization incompetent Mad2^{RQ} (Figure 37B, lanes 13 versus 15; Figure 38B,C,D,E). Sucrose gradient sedimentation of the complexes released from the initial antibody coated beads confirmed a 2-3 fold, chromosome-dependent increase in BubR1/Bub3 bound to APC/C (Figure 40). Thus, Bub3/BubR1 binding to APC/C is facilitated by dimerization competent Mad2 and unattached kinetochores through a mechanism that does not affect Cdc20 interaction with APC/C.

A soluble kinetochore-derived Cdc20-BubR1-Bub3 inhibitor.

To determine the composition(s) of complexes produced spontaneously or by action of unattached kinetochores in the absence of APC/C, equal stoichiometries of various combinations of BubR1, Bub3, Mad2, and Cdc20 were incubated with or without purified chromosomes, the chromosomes were removed and the inhibitor-containing supernatant (as in Figure 33) was subjected to size exclusion chromatography. As expected, Bub3 shifted into a larger complex, co-eluting with BubR1 under all conditions. Most Cdc20 shifted into a substantially higher molecular weight complex both in the presence (Figure 41E) and absence (Figure 41D) of chromosomes, eluting together with BubR1 and Bub3 (centered on fractions 12 and 13). Except for a small proportion of Mad2 bound to Cdc20 when incubated alone with it (Figure 41C), surprisingly little Mad2 chromatographed with Cdc20 under any condition, eluting instead at a position corresponding to a Mad2 monomeric form, regardless of the presence of chromosomes and/or BubR1/Bub3. Very little Mad2 was found in an MCC-like tetrameric complex with BubR1, Bub3, and Cdc20 in the absence of chromosomes, even when using conditions that produced up to 80% inhibition of Cdc20's ability to activate APC/C (Figure 15C). Incubation with chromosomes eliminated even this small amount of MCC-like complex (Figure 41D,E). On the other hand, incubation with chromosomes produced a proportion of BubR1 and Cdc20 that eluted earlier (e.g., fraction 11), consistent with production of a larger complex or one with a more extended structure so as to produce a higher Stoke's radius. This

complex was comprised of approximately equal molar amounts of BubR1 and Cdc20, but only trace levels of Mad2.

Discussion:**Unattached kinetochores amplify APC/C inhibition by promoting the association of BubR1 with APC/C^{Cdc20}.**

Production of at least two inhibitors can be enhanced by unattached kinetochores: one containing diffusible Cdc20 and another in which Cdc20 is already bound in a megadalton complex to APC/C, consistent with reports that Cdc20 and checkpoint proteins are present in two complexes with differing sizes during mitosis (Braunstein et al., 2007; Morrow et al., 2005; Sudakin et al., 2001; Wang et al., 2006). Both inhibitors prevent recognition by APC/C of cyclin B as an ubiquitination substrate. Disruption of cyclin B ubiquitination by a kinetochore-derived inhibitor even while Cdc20 remains bound to APC/C provides a potential explanation for the differential timing of destruction of cyclins A and B. Instead of simple sequestration of Cdc20, a kinetochore-derived mitotic checkpoint inhibitor bound to APC/C^{Cdc20} may block recognition of cyclin B as an ubiquitination substrate, while permitting APC/C^{Cdc20}-mediated ubiquitination and destruction of cyclin A, an event that is known to initiate immediately after mitotic entry (den Elzen and Pines, 2001; Geley et al., 2001). Additionally, we found no requirement for direct contact of the APC/C with unattached kinetochores to amplify its inhibition. While it has previously been argued that the kinetochore may sensitize the APC/C for

checkpoint-mediated inhibition (Acquaviva et al., 2004; Sudakin et al., 2001), our data did not support that inference.

Despite amplification of Cdc20 inhibition when equal molar levels of BubR1, Mad2 and Cdc20 were added, we found no evidence for assembly of a quaternary MCC-like complex as a *bona fide* inhibitor produced by unattached kinetochores either. Rather, almost all Cdc20 shifted to a complex co-migrating with the majority of BubR1, but containing very little Mad2 (Fig. 41E). Also arguing against a contribution in kinetochore-derived checkpoint signaling, we note that the reported MCC-like complexes *in vivo* are present outside of mitosis (Fraschini et al., 2001; Sudakin et al., 2001), and their formation in yeast continues in the absence of a functional centromere/kinetochore (Fraschini et al., 2001). All of this supports an MCC-like, premade Cdc20 inhibitor produced in a kinetochore-independent manner in interphase that restrains APC/C ubiquitination activity for cyclin B just after mitotic entry, which has been referred to as a “timer” (Meraldi et al., 2004).

A model for mitotic checkpoint signaling: kinetochore produced Mad2-Cdc20 as a precursor to BubR1-Cdc20 as sequentially produced mitotic checkpoint inhibitors.

Our evidence supports unattached kinetochores acting on Mad2 in catalyzing amplification of a mitotic checkpoint inhibitor for blocking APC/C^{Cdc20} recognition of cyclin B as a substrate for ubiquitination, with

BubR1 acting downstream of Mad2 activation (Chapter 2). The majority of the cellular Mad2, which is present at relatively equimolar concentrations to Cdc20 during mitosis (Tang et al., 2001), remains in the open conformation that is less capable of interacting with Cdc20 (Luo et al., 2004). Moreover, incubation of physiologically relevant concentrations of each component produced most Cdc20 bound to BubR1, not Mad2, whether or not chromosomes were present (Figure 41D,E) and with unattached kinetochores both lowering the amount of Mad2 associated with a Cdc20-containing complex(es) and generating a Bub3-BubR1-Cdc20 complex eluting at a higher Stokes radius.

We propose from all of this a model (Figure 42) in which Mad1/Mad2 immobilized at kinetochores templates conversion of an inactive, open Mad2 to one capable of transient capture of Cdc20, followed by relay to BubR1 as sequentially produced mitotic checkpoint inhibitors. Incubation with unattached kinetochores and the corresponding amplification of inhibition of Cdc20 for activating APC/C is accompanied by a shift to a more rapidly eluting Bub3/BubR1-Cdc20 complex without a stable pool of Mad2-Cdc20. This evidence supports Mad2-Cdc20, and perhaps an MCC-like complex, as a transient intermediate in kinetochore-mediated checkpoint signaling and one that is a precursor to BubR1-Cdc20. Further, Bub3/BubR1 binds to APC/C but only in a Mad2-dependent manner that is stimulated by unattached kinetochores (Figure 37), demonstrating that kinetochores facilitate loading of Bub3/BubR1 onto APC/C. That BubR1-APC/C^{Cdc20} is produced indirectly by

unattached kinetochores as the final Cdc20 inhibitor would also support suggestions that BubR1 acts as a non-productive pseudosubstrate of the APC/C (Burton and Solomon, 2007) or mediates Cdc20 proteolytic turnover (King et al., 2007; Pan and Chen, 2004).

Combining kinetochore-derived Bub3/BubR1-Cdc20 with evidence for two Cdc20 binding sites on BubR1 (Davenport et al., 2006) further suggests that the spontaneous and kinetochore derived Bub3/BubR1-Cdc20 complexes may represent generation of Cdc20 bound at the two different sites respectively, a point now testable with the appropriate BubR1 mutants.

Materials and Methods:

Protein Purifications. Proteins were purified as described in Chapter 2.

APC/C Ubiquitination Assays. Ubiquitination assays were performed as described in Chapter 2.

GST-Cdc20 Pull Down Assays. GST-tagged Cdc20 was purified and incubated with recombinant proteins for one hour in storage buffer (Chapter 2). Glutathione-Sepharose beads were used to isolate GST-Cdc20 and bound components, which were then separated from the supernatant by gentle centrifugation. The beads were washed twice in storage buffer and GST-Cdc20 was eluted by the addition of 100nM reduced glutathione. The whole of the elution was separated from the beads by strong centrifugation, boiled, and subjected to immunoblot analysis.

APC/C Complex Affinity, Elution, and Sucrose Sedimentation. APC/C was immunoprecipitated using a peptide-derived Cdc27 antibody (Herzog and Peters, 2005) conjugated to Affiprep Protein A (BioRad) beads for 2 hours from *Xenopus* interphase extracts. The washed APC/C beads were incubated with recombinant checkpoint proteins for 1hr at room temperature. The beads were washed twice with 20 volumes TBS buffer to remove unbound proteins. The APC/C complex was eluted from the beads by Cdc27 peptide competition as previously described (Herzog and Peters, 2005) and analyzed by immunoblotting. Sucrose sedimentation of APC/C complexes was performed

by scaling up APC/C elution five-fold and placing the eluate over a 5-35% sucrose gradient. The gradients were spun for 4 hours at 50,000rpm speed in a Beckman tabletop ultracentrifuge with TLS55 swinging bucket rotor. The fractions were collected from top to bottom and TCA precipitated before being subjected to immunoblot analysis.

Gel Filtration of Complexes of Mad2, BubR1, Bub3 and Cdc20. Various equimolar combinations of BubR1, Bub3, Cdc20, and Mad2 proteins were incubated with or without purified mitotic chromosome for 30 min at room temperature and protein complexes generated were resolved on Superose 6 gel filtration column chromatography. Proteins in each column fraction were concentrated by precipitation with trichloroacetic acid (TCA) and further analyzed by immunoblotting.

The text of Chapter 2 and 3, in part and with modifications, will be published in Developmental Cell, 2009, as a single manuscript. I was the primary researcher and author of this work. Joo Seok Han also contributed to this work. Don Cleveland directed and supervised the research that forms the basis of these chapters.

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Figure 30. Models of APC/C activation by Cdc20.

Cdc20 has been proposed to activate APC/C either by **(A)** a stoichiometric interaction through the formation of a stable complex, or **(B)** a transient interaction.

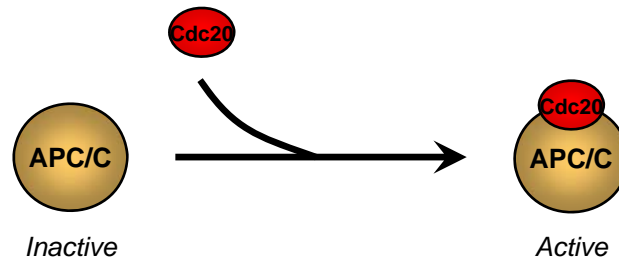
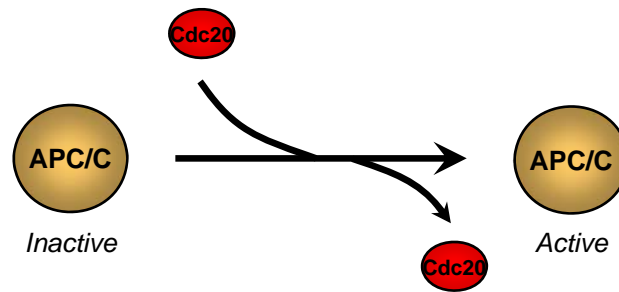
A**B**

Figure 31. Models of APC/C inhibition by checkpoint protein complexes.

APC/C has been proposed to be inactivated in mitosis by **(A/B)** the formation of complexes which sequester Cdc20 away from the APC/C, thereby preventing **(A)** the formation of a Cdc20-APC/C complex or **(B)** the transient interaction of Cdc20 with the APC/C. **(C/D)** The binding of checkpoint proteins to Cdc20 while it remains associated with the APC/C have also been suggested, with **(D)** the possibility that checkpoint proteins dissociate Cdc20 from the APC/C complex.

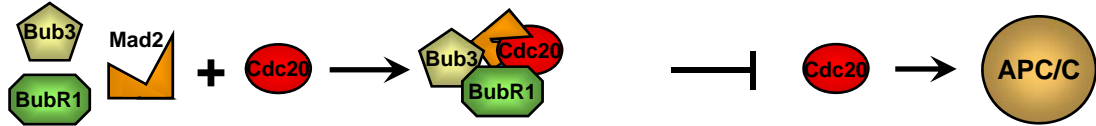
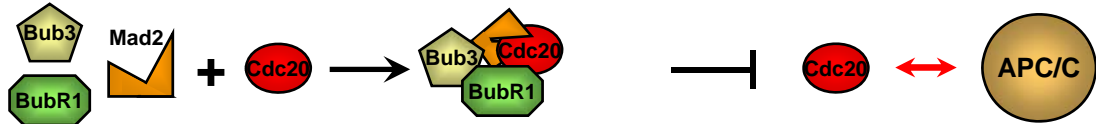
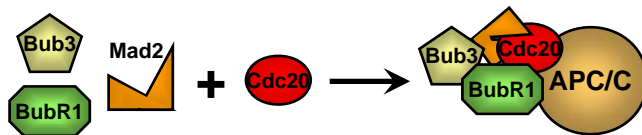
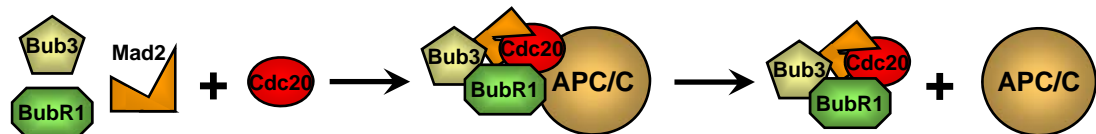
A**Sequestration****B****Sequestration w Transient Activation****C****Large Complex Formation****D****Large Complex Formation with Dissociation**

Figure 32. Models of kinetochore mediated transmission of a checkpoint active state.

Models of kinetochore dependent checkpoint signaling have proposed that **(A)** kinetochores serve as the site of assembly of inhibitory complexes which inactivate APC/C^{Cdc20}, or **(B)** kinetochores sensitize APC/C for inhibition by pre-made inhibitors.

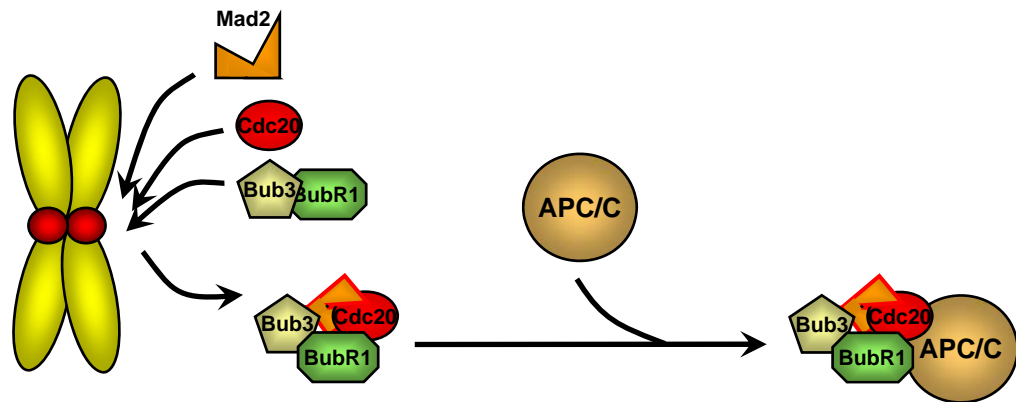
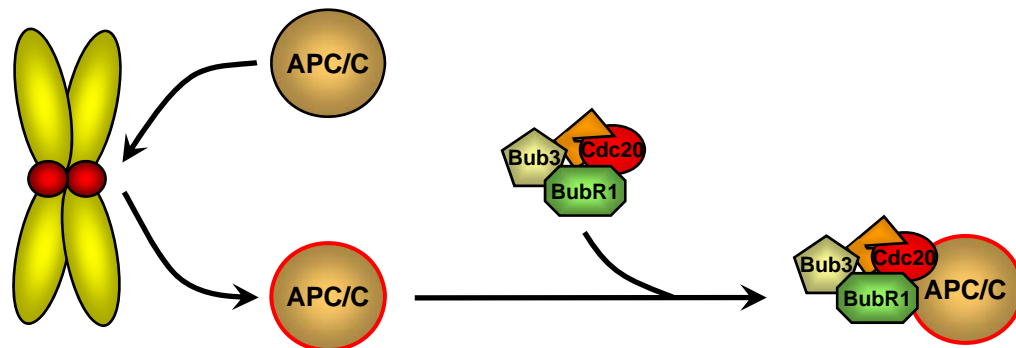
A**B**

Figure 33. Production of a diffusible APC/C^{Cdc20} inhibitor by unattached kinetochores.

(A) Chromosomes were incubated with BubR1, Bub3, Mad2, and Cdc20 (1:1:1:5); the chromosomes were subsequently removed by centrifugation, and (blue) the supernatant fraction was **(B)** assayed for activation of APC/C for cyclin B ubiquitination. (Red) Parallel assay was done without chromosome removal or (white) without initial chromosome addition. **(C)** Results were quantified below.

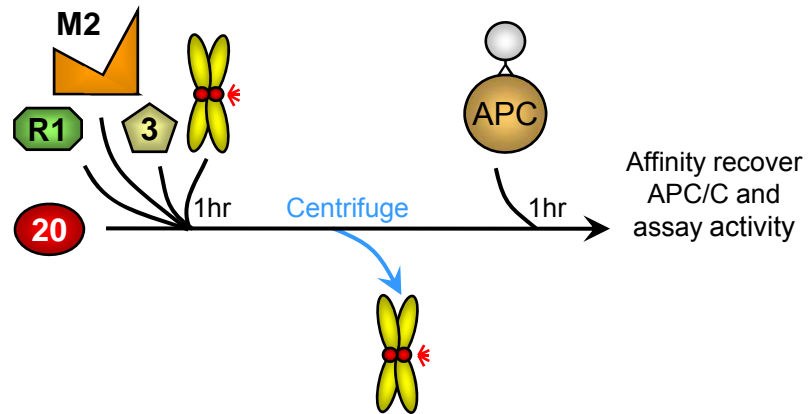
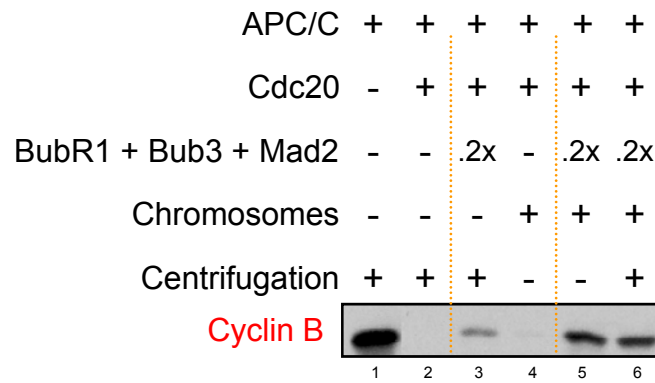
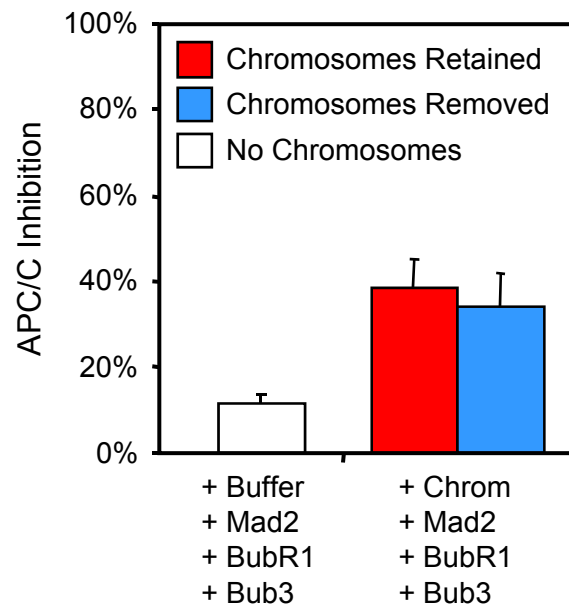
A**B****C**

Figure 34. Checkpoint inhibitors do not determine cyclin specificity of APC/C^{Cdc20}.

(A) Cyclin A was substituted for Cyclin B as a substrate for APC/C ubiquitination. **(B)** Purification of cyclin A¹⁻¹⁰², cyclin A⁹⁸⁻⁴³², and full length cyclin A substrates, assessed by Coomassie staining. **(C)** Incubation of Cdc20 or Cdh1 with APC/C stimulates ubiquitination of cyclin A substrates. **(D)** Addition of BubR1, Bub3, Mad2, and chromosomes to Cdc20 blocks the ubiquitination of cyclin A by either interphase or mitotic APC/C.

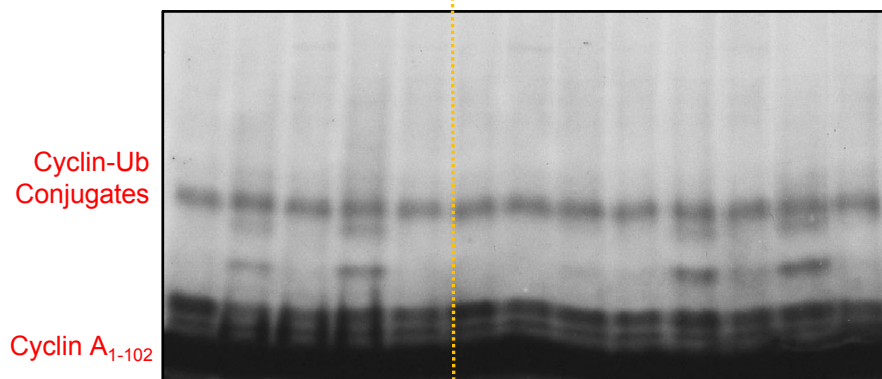
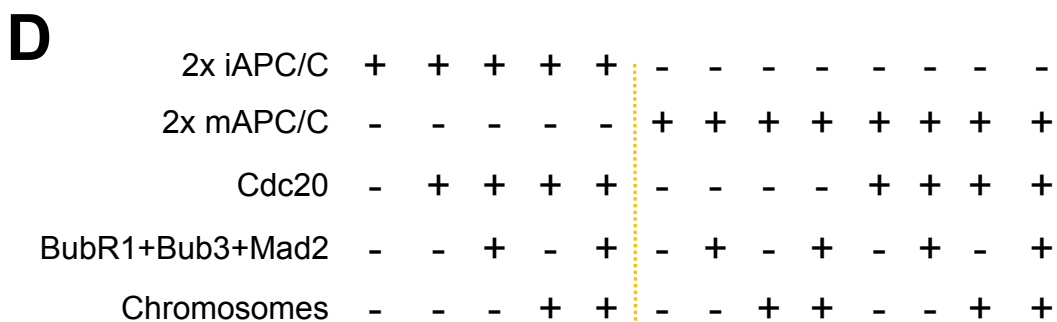
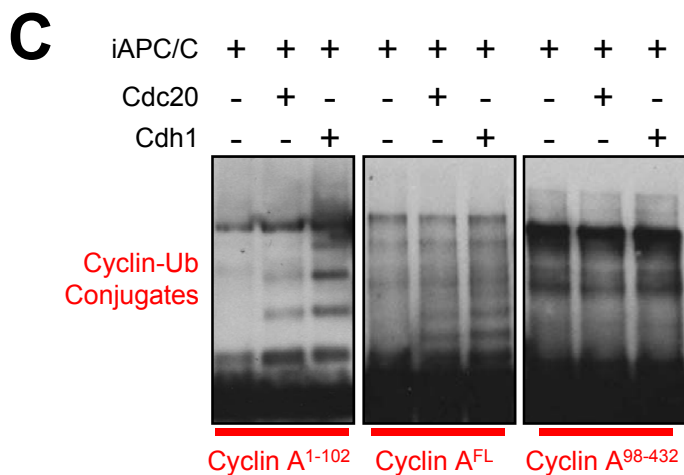
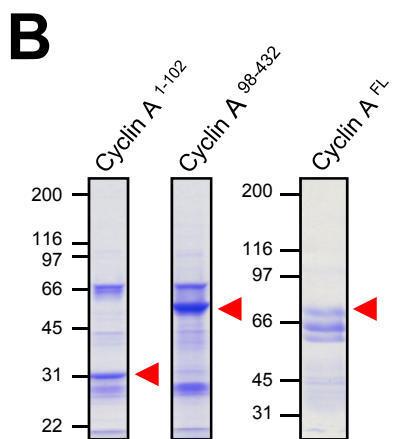
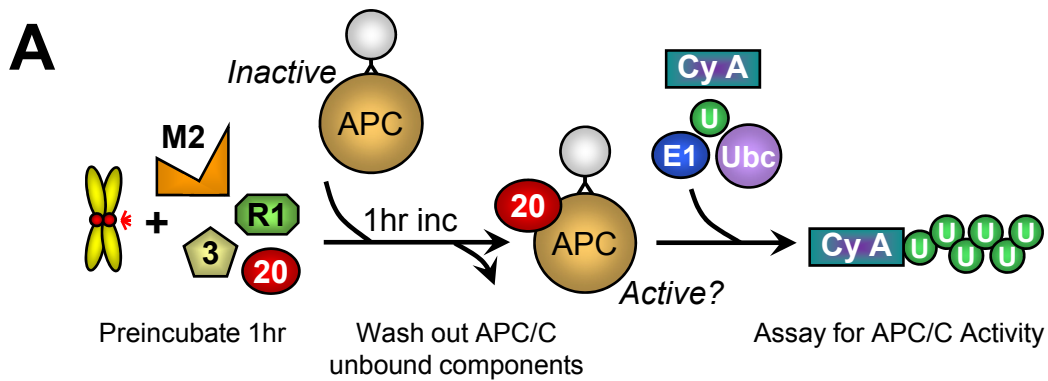


Figure 35. Inhibition of APC/C activation is not achieved solely by sequestration of Cdc20.

(A) Checkpoint components were co-incubated together with Cdc20 and chromosomes, followed by APC/C addition and assay for its activity (C). **(B)** Immunoprecipitated APC/C was first incubated with Cdc20 to form an active complex ("Pre-activated APC/C"). APC/C was affinity recovered and subsequently incubated with chromosomes and increasing amounts of BubR1, Bub3, and Mad2, and APC/C activity was assayed **(C)**. **(D)** Quantitation of (red triangles) co-incubated and (blue squares) pre-activated Cdc20-stimulated APC/C activity.

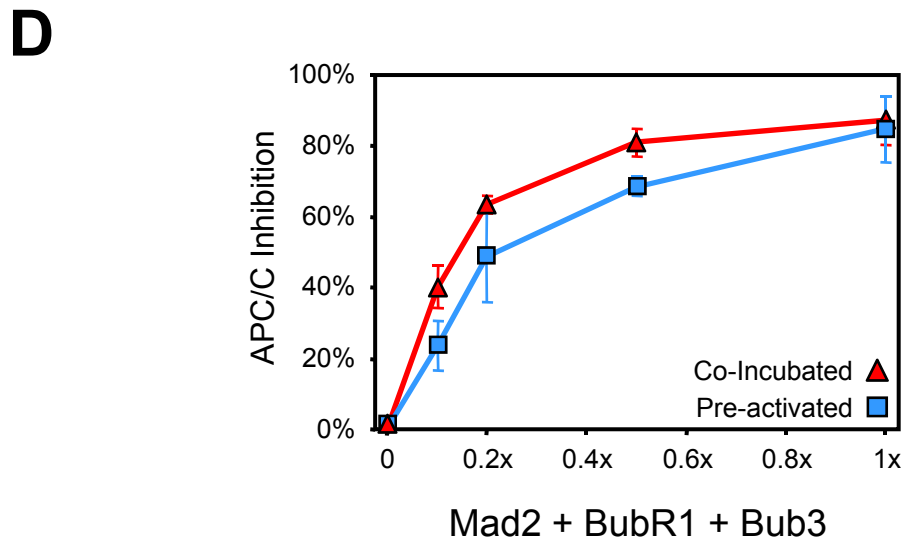
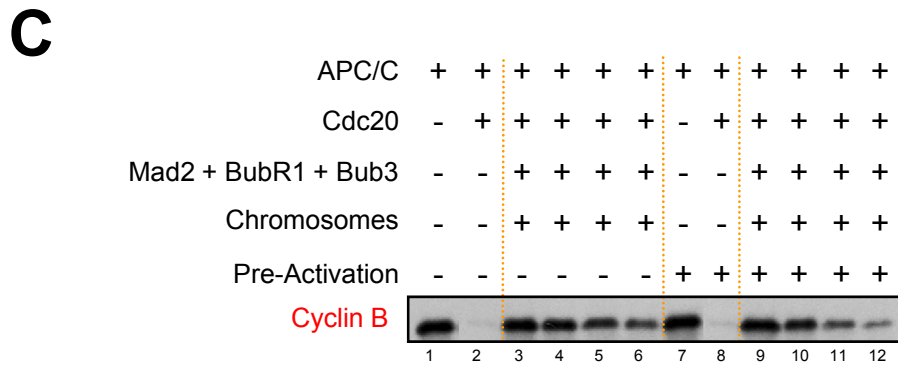
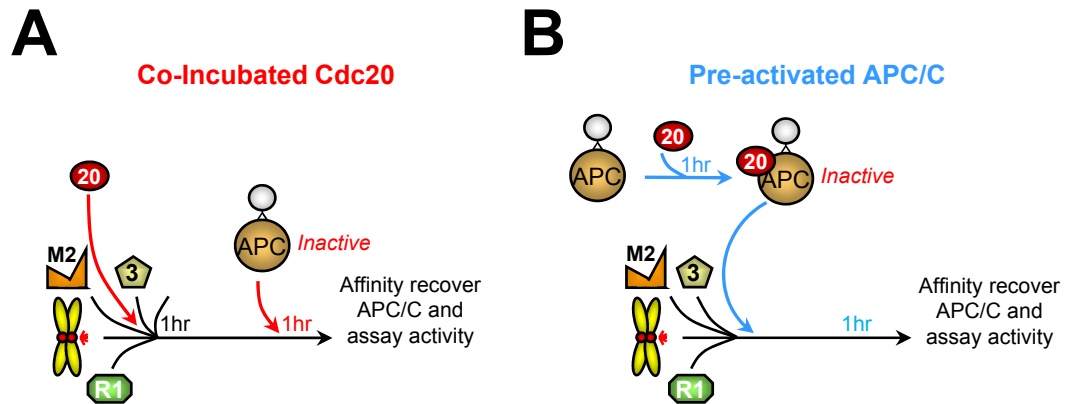


Figure 36. BubR1 binds free Cdc20.

(A) BubR1 was incubated with Cdc20 fused to GST, in the presence or absence of Mad2 and chromosomes. Cdc20 containing complexes were recovered by glutathione affinity, washed, eluted by reduced glutathione, and **(B)** analyzed by immunoblotting for Cdc20, BubR1, Bub3, and Mad2. BubR1 bound to free Cdc20 without the presence of Mad2 or unattached kinetochores being needed.

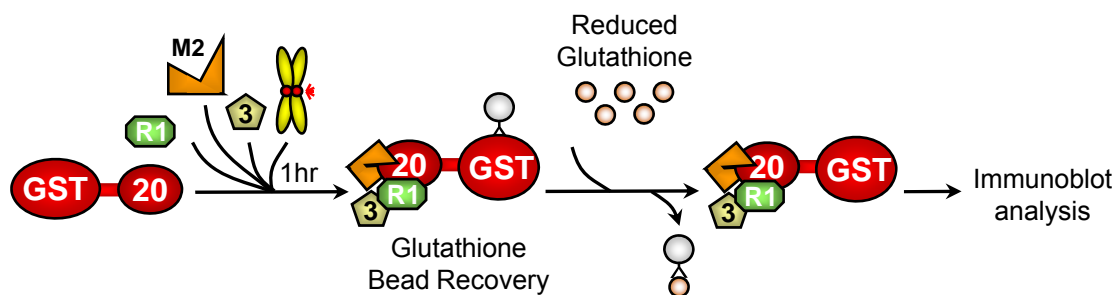
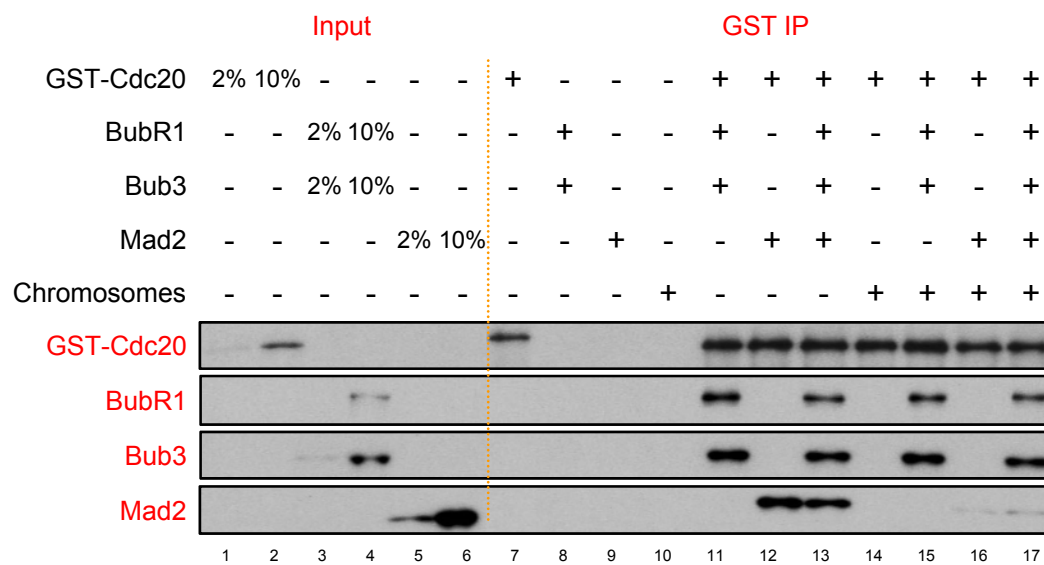
A**B**

Figure 37. BubR1 and Bub3 association with APC/C^{Cdc20} is facilitated by Mad2 and unattached kinetochores.

(A) APC/C was incubated with pre-incubated combinations of Cdc20, Mad2, BubR1, Bub3 and chromosomes, recovered, peptide-eluted from Affiprep beads, and **(B)** analyzed for bound components by immunoblotting. BubR1 binding to APC/C^{Cdc20} was mediated by Mad2, and further amplified in the presence of unattached kinetochores.

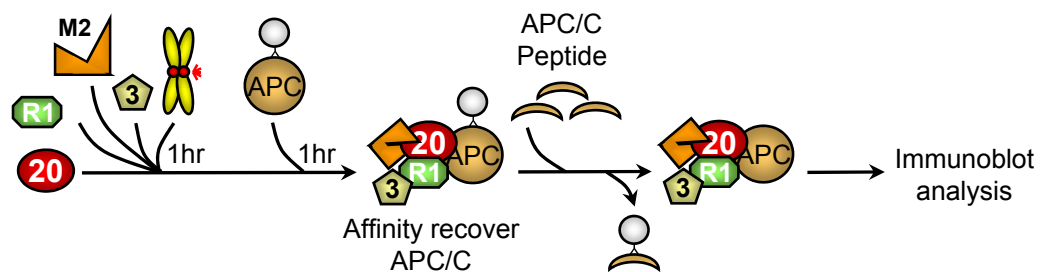
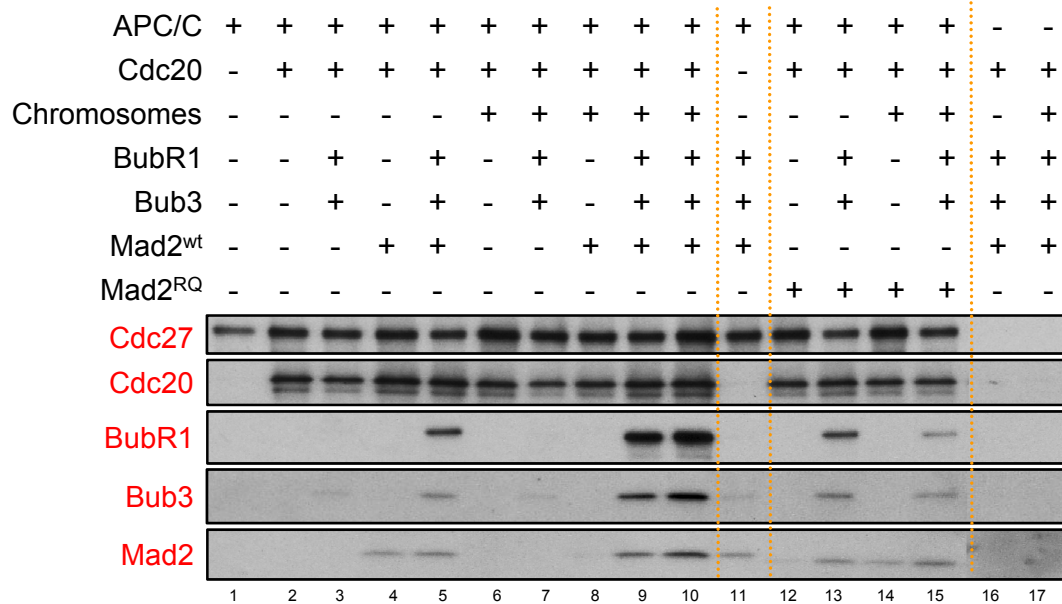
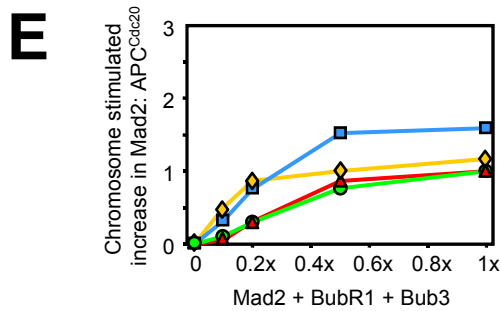
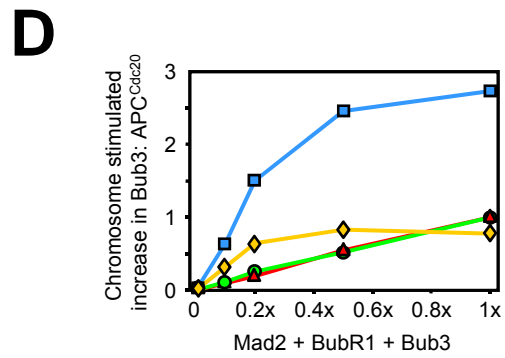
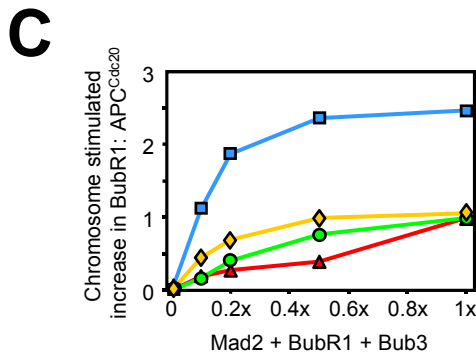
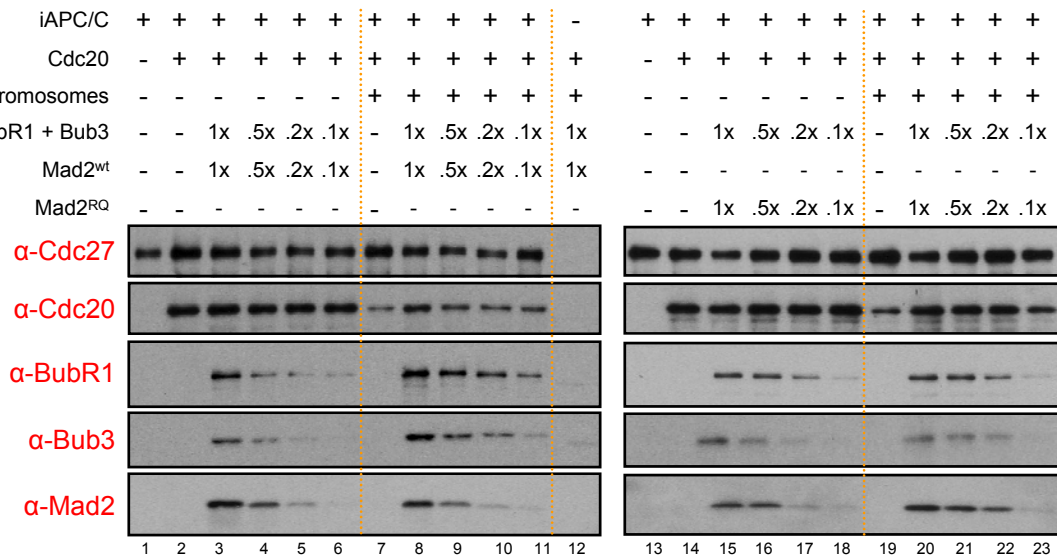
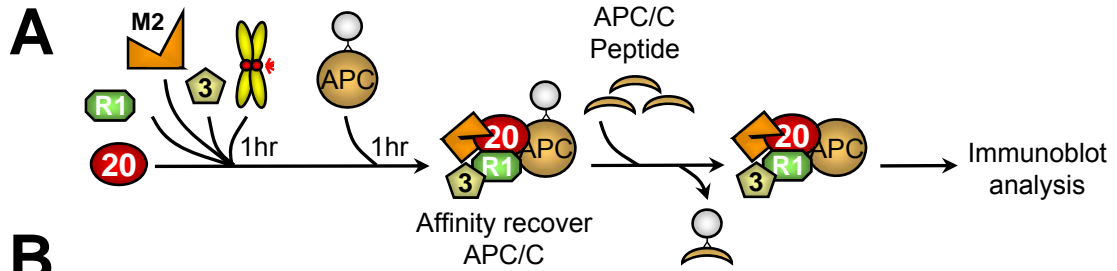
A**B**

Figure 38. Unattached kinetochores facilitate BubR1, Bub3, and Mad2 association with APC/C^{Cdc20}.

(A) APC/C was incubated with Cdc20 and increasing amounts of BubR1, Bub3, and Mad2^{wt} (or Mad2^{RQ}) either in the presence or absence of chromosomes, treated as in Figure 37, and **(B)** analyzed by immunoblotting. The amounts of eluted **(C)** BubR1, **(D)** Bub3, and **(E)** Mad2^{wt} (or Mad2^{RQ}) were measured against a dilution series of purified protein and quantified relative to the amount of Cdc20 bound to APC/C. Values were plotted as fold change over the initial (1x) non-chromosome incubated eluted amounts.

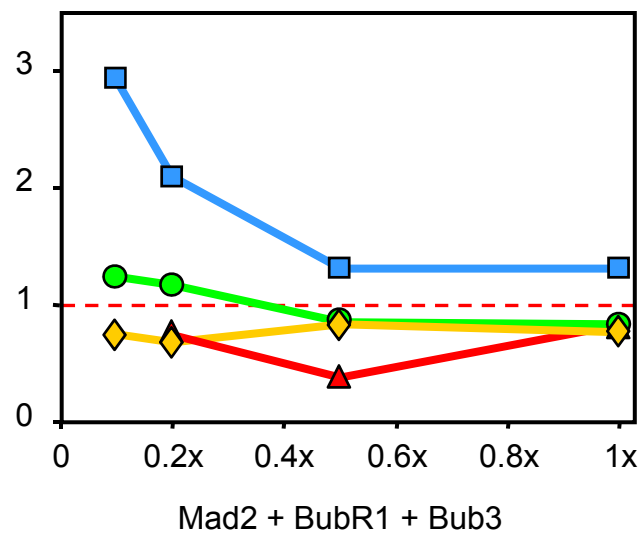


▲ + Mad2^{wt} + BubR1 + Bub3 + Buffer ● + Mad2^{RQ} + BubR1 + Bub3 + Buffer
■ + Mad2^{wt} + BubR1 + Bub3 + Chromosomes ◆ + Mad2^{RQ} + BubR1 + Bub3 + Chromosomes

Figure 39. Substoichiometric amounts of Mad2 relative to BubR1 are found associated with APC/C^{Cdc20}.

Relative stoichiometry of BubR1 molecules to Mad2 molecules associated with the APC/C complex. BubR1 relative stoichiometry to Mad2 increased above 1:1 when chromosomes were present, but remained approximately 1:1 or below when Mad2^{wt} was replaced with Mad2^{RQ} regardless of the presence of chromosomes.

Molecules BubR1:Mad2 bound to APC/C^{Cdc20}



- ▲ + Mad2^{wt} + BubR1 + Bub3 + Buffer
- + Mad2^{wt} + BubR1 + Bub3 + Chromosomes
- + Mad2^{RQ} + BubR1 + Bub3 + Buffer
- ◆ + Mad2^{RQ} + BubR1 + Bub3 + Chromosomes

Figure 40. Unattached kinetochores facilitate BubR1, Bub3, and Mad2 association with Cdc20 bound to the APC/C complex.

(A) APC/C incubated with combinations of Cdc20, Mad2, BubR1, Bub3 and chromosomes was recovered, eluted from Affiprep beads, fractionated over a sucrose gradient, and **(B)** analyzed by immunoblotting for bound components.

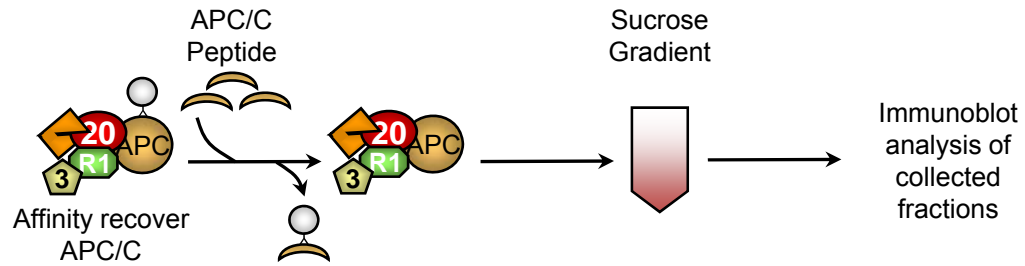
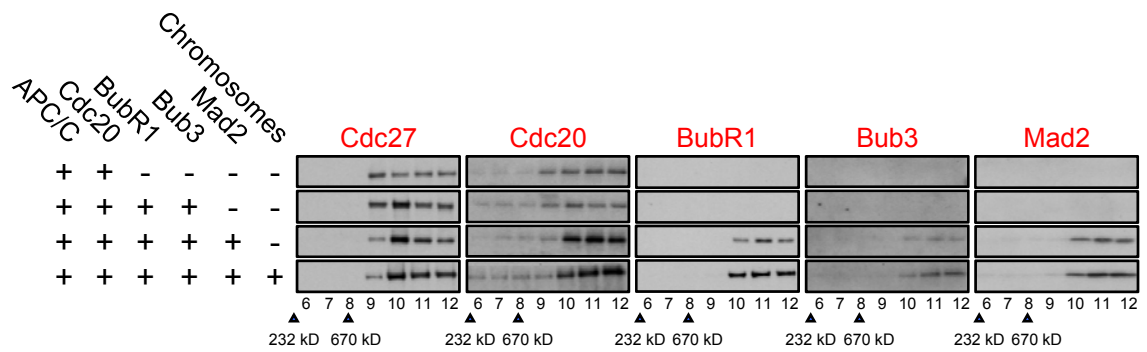
A**B**

Figure 41. Kinetochores catalyze the production of a BubR1-Cdc20 inhibitor without stably associated Mad2.

(A) Individual components or **(B-E)** combinations of Cdc20, Mad2, BubR1, Bub3, and/or chromosomes were incubated for 1hr and subsequently fractionated over a Superose-6 filtration column. **(E)** Mixtures containing chromosomes were first centrifuged to pellet the chromosomes prior to loading onto the column. Fractions eluted from the column were analyzed for BubR1, Bub3, Cdc20, and Mad2 content by immunoblotting for those components.

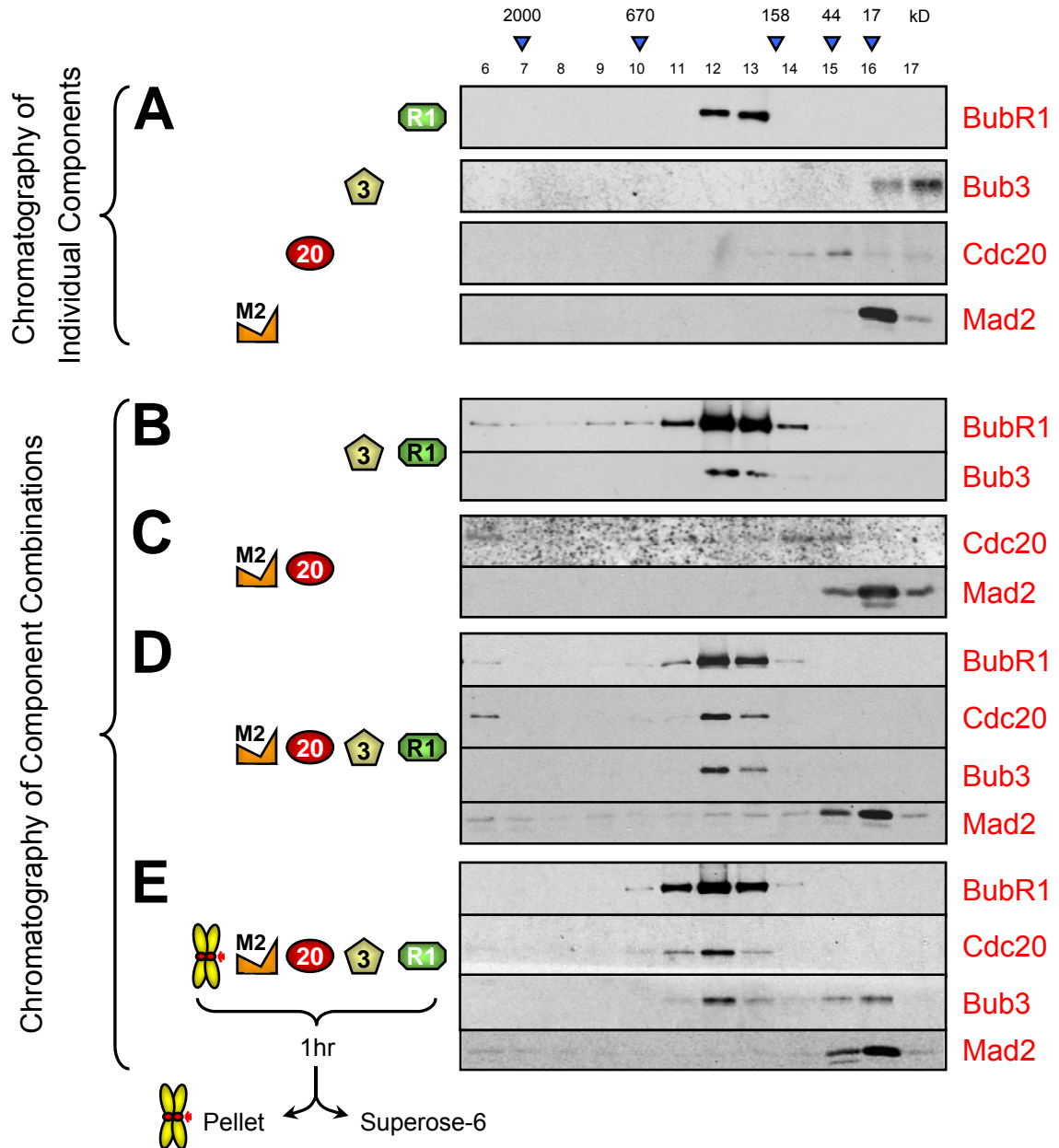
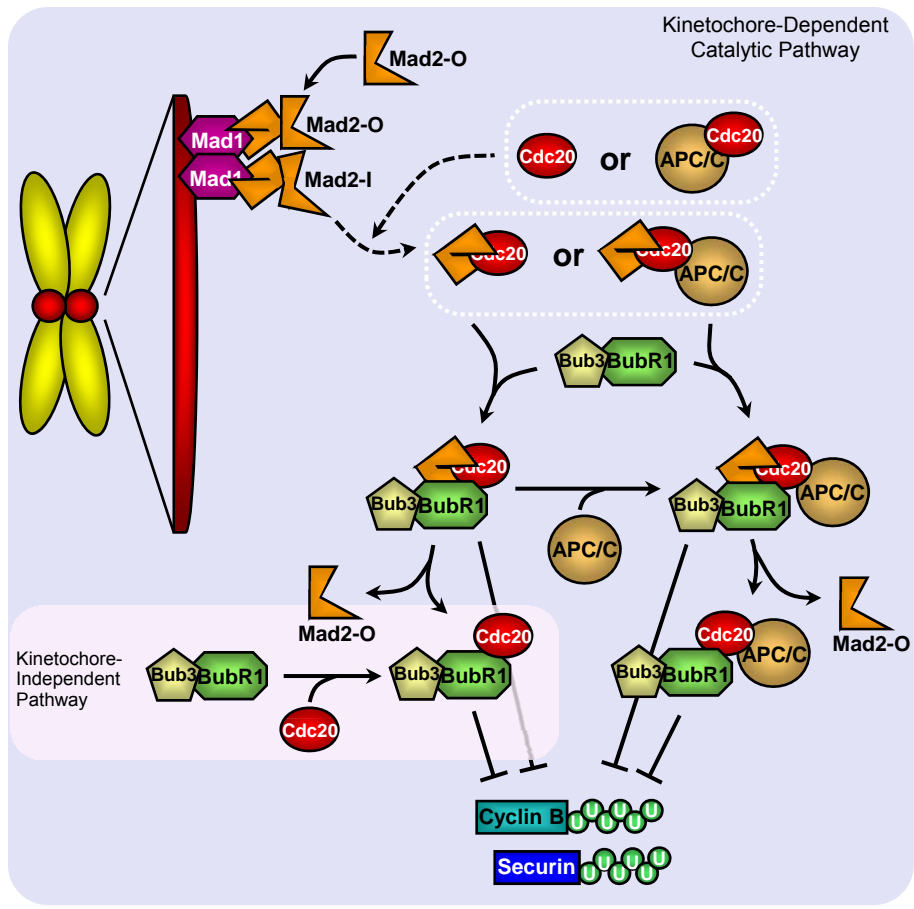


Figure 42. Model for generation of a “wait anaphase” mitotic checkpoint inhibitor by sequential production of Mad2-Cdc20 and BubR1-Cdc20 inhibitors.

Cytosolic Mad2 in an initially open conformation is recruited to unattached kinetochores via an immobilized Mad1:Mad2 heterodimer. This second molecule of Mad2 binds in an activated conformation that is poised for capture of Cdc20 either while kinetochore bound or after release. This transient Mad2-Cdc20 complex promotes handoff of Cdc20 to BubR1, thereby inhibiting ability of that Cdc20 to activate ubiquitination by APC/C of cyclin B, both by sequestering Cdc20 from APC/C and by inhibiting Cdc20 while APC/C bound.



CHAPTER 4.

Implications of Cdc20 Binding to BubR1

Anita Kulukian, Joo Seok Han and Don Cleveland

Abstract:

BubR1 is a multidomain protein essential for the mitotic checkpoint. Aside from its GLEBS and kinase domains, it contains two Cdc20 binding sites. Using differentially tagged Cdc20 protein pull-down assays, a single BubR1 molecule is demonstrated to bind to two distinct molecules of Cdc20. The amino terminal Cdc20 binding domain of BubR1 becomes a potent inhibitor of the APC/C solely when Mad2 is present. This is likely not the case with the internal Cdc20 binding site, which is predicted to bind Cdc20 without Mad2 mediation. These differentially regulated Cdc20 binding domains of BubR1 may represent two different pathways of Cdc20 inhibition, with one corresponding to checkpoint-activated kinetochore-dependent signaling, while the other potentially signifying a kinetochore-independent pathway to keep APC/C activity in check prior to mitotic entry and the activation of unattached kinetochore signaling.

Introduction:

BubR1, a protein essential for the mitotic checkpoint, is composed of multiple functional domains. It contains a 20-amino acid motif (known as the GLEBS domain) which is required for its interaction with Bub3 and mediates its localization to kinetochores (Taylor et al., 1998). Its carboxy terminal encompasses a kinase domain, which is activated by CenpE, and is required for its mitotic function (Chan et al., 1999; Mao et al., 2003; Mao et al., 2005). BubR1 also plays a significant role in inhibiting APC/C activation during metaphase prior to bipolar chromosome attachment. This function of BubR1 is mediated through its binding of the APC/C activator Cdc20 (Tang et al., 2001).

Cdc20 binding region of BubR1 was initially characterized by assaying inhibitory capacity of BubR1 truncation mutants towards APC/C ubiquitination activity *in vitro* in the absence of additional inhibitory factors or unattached kinetochores. The Cdc20 binding region was localized between residues 526-700, though with the caveat that there could be multiple Cdc20 binding sites on BubR1: a truncation mutant spanning 351-700 could bind to Cdc20 but could not inhibit APC/C. In contrast, the amino terminus was demonstrated to be incapable of inhibiting cyclin B ubiquitination. Proceeding work suggested that the amino terminal of BubR1 may have additional roles in mediating its checkpoint functions (Harris et al., 2005), likely through the presence of an additional Cdc20 binding site in the amino terminal 477 amino acids that has a

propensity for binding Mad2-Cdc20 (Davenport et al., 2006). Work contemporary to ours demonstrated that overexpression of the amino terminus (the first 363 a.a.) of BubR1 helps sustain mitotic arrest in response to nocodazole in MEFs homozygous deleted for BubR1 more strongly than the carboxy terminal of BubR1 (a.a. 363-1052) (Malureanu et al., 2009).

The presence of potentially two different Cdc20 binding sites within BubR1 has raised additional questions about which site, if not both, is functional in Cdc20 binding. It raises the possibility that each site may bind to two separate Cdc20 molecules or that the two sites are needed to bind Cdc20 in a more inhibitory conformation. It also raises questions about the functionality and checkpoint regulation of each site, questions which we begin to elucidate here.

Preliminary Results:**BubR1 can bind to two distinct molecules of Cdc20.**

To determine whether BubR1 bound to two molecules of Cdc20, BubR1 was incubated with two differentially tagged purified Cdc20. Cdc20 fused to GST can be distinguished from the HIS-tagged version by a corresponding decrease in gel mobility indicative of an increase in size. GST-Cdc20 bound complexes were isolated by affinity for glutathione conjugated beads. Both HIS-Cdc20 and GST-Cdc20 can form complexes with BubR1 (Figure 41,43C lane 8), the amount of which increases in the presence of Mad2 (Figure 43C, lane 9 versus 12, lane 10 versus 11). When BubR1 was incubated with both GST-Cdc20 and HIS-Cdc20, HIS-Cdc20 precipitated within the complex of GST-Cdc20-BubR1. Since HIS-Cdc20 and GST-Cdc20 do not form a complex without BubR1 (Lane 7), we conclude that a single molecule of BubR1 can bind to two distinct molecules of Cdc20.

The amino-terminal Cdc20 binding site of BubR1 inhibits Cdc20 only in the presence of Mad2.

To determine whether the BubR1 amino-terminal binding site of Cdc20 was a bona-fide inhibitory domain, it was tested for competency in inhibiting APC/C ubiquitination activity. BubR1 truncation mutants encompassing the amino-terminal (1-363) and internal (357-1050) binding domains of Cdc20

were cloned and purified. Addition of Cdc20 to increasing concentrations of BubR1¹⁻³⁶³ could not inhibit the ubiquitination of Cyclin B (Figure 44, red triangles). However, the addition of Mad2 to those incubations produced robust inhibition of APC/C^{Cdc20} activity by BubR1¹⁻³⁶³ (Figure 44, blue squares). Thus Mad2 regulates BubR1¹⁻³⁶³ inhibition of Cdc20.

Discussion:**Two differentially regulated Cdc20 binding sites of BubR1 may represent a checkpoint dependent and independent pathways of APC/C regulation.**

BubR1 binding to APC/C^{Cdc20} inhibits premature prometaphase activation of APC/C (Tang et al., 2001). It is now apparent that two regions of BubR1 are responsible for its binding to Cdc20. Rather than requiring both sites to bind to an individual Cdc20 molecule, the two regions seem to function independently to bind to two distinct Cdc20 molecules (Figure 43).

The regulation of the two Cdc20 binding sites of BubR1 seems to be distinct as well. While the amino terminal site requires Mad2 to enable its interaction with Cdc20, the internal Cdc20 binding sites may be capable of binding directly to Cdc20 (a point which should be tested directly with the availability of the BubR1 internal truncation mutant). Full length BubR1 thus seems to be the composite of the function of both Cdc20 binding sites; some BubR1 binds to Cdc20 without Mad2, but its binding is increased in the presence of Mad2 (Figure 43). Similarly, BubR1 can somewhat inhibit APC/C^{Cdc20} independent of Mad2 and unattached kinetochores (Figure 15B). BubR1 becomes a more potent inhibitor in the presence of Mad2, and can inhibit APC/C almost to completion only when chromosomes are incubated with Mad2 (Figure 15C).

Differential regulation of BubR1 Cdc20 binding may represent two different pathways regulating APC/C^{Cdc20} converging upon one molecule. The amino-terminal Cdc20 binding site of BubR1 is likely to be checkpoint regulated. Because its binding to and inhibition of Cdc20 is mediated by Mad2, the molecule which is directly activated by unattached kinetochores (Chapter 2), BubR1¹⁻³⁶³ inhibition of Cdc20 is strongly suggestive of a kinetochore-dependent mechanism regulating BubR1 activity. The internal Cdc20 site, on the other hand, which is likely to bind Cdc20 without Mad2, may be representative of a kinetochore-independent pathway in action. It would explain why BubR1 and Cdc20 containing complexes can be found in yeast in the absence of a functional kinetochore (Fraschini et al., 2001) and it would explain the presence of premade APC/C inhibitors prior to mitotic entry (Meraldi et al., 2004; Sudakin et al., 2001). Many additional experiments remain to be completed, including the validation of Mad2- and thus checkpoint-independent regulation of BubR1's internal Cdc20 binding site, but should prove extremely interesting in terms of a novel BubR1 regulatory mechanism.

Materials and Methods:

Protein Purification. The amino terminal of BubR1 was cloned by the PCR amplification of residues encompassing 1 to 363. Similarly, the internal Cdc20 binding site spanning residues 357-1050 was amplified by PCR. PCR fragments were cloned into pFastBac GST-tagged expression vector, which was used to generate baculovirus for insect cell infection. Proteins were purified as described for GST-tagged proteins in Chapter 2, with the additional steps of PreScission protease digestion for GST tag removal and further purification over Superose 6 column.

GST-Cdc20 Pull Down Assays were performed as described in Chapter 3.

APC/C Ubiquitination Assays were performed as described in Chapter 2.

The text of Chapter 4 will be submitted for publication once the research has been completed. Joo Seok Han and I contributed equally to the research for this project, while I was the primary author. Don Cleveland directed and supervised the research that forms the basis of Chapter 4.

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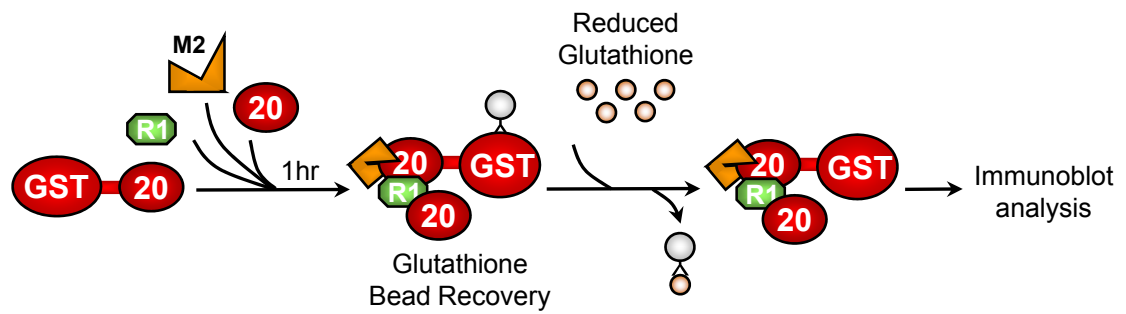
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Figure 43. BubR1 is a modular protein which binds to two molecules of Cdc20.

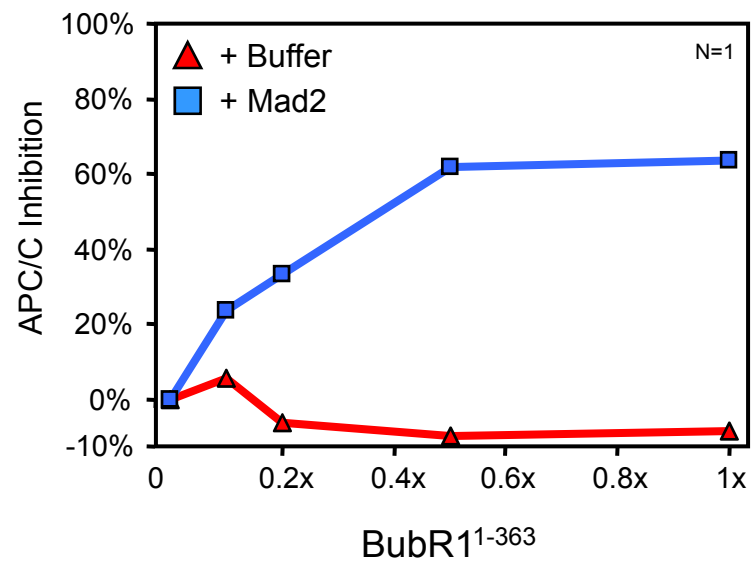
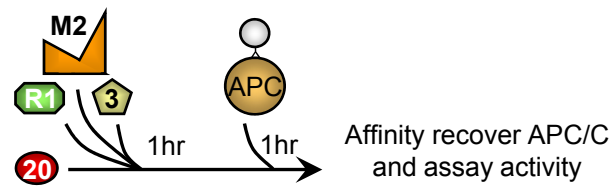
(A) Schematic representation of human BubR1 modular domains. **(B)** BubR1 was incubated with GST-Cdc20 and HIS-Cdc20, in the presence or absence of Mad2. GST-Cdc20 containing complexes were recovered by glutathione affinity, washed, eluted by reduced glutathione, and **(C)** analyzed by immunoblotting for Cdc20, BubR1, Bub3, and Mad2. BubR1 binding to both HIS-Cdc20 and GST-Cdc20 in a single complex was mediated by Mad2.

A**BubR1****B****C**

	2.5% Input				Anti-GST IP								Mock IP		
GST-Cdc20	+	-	-	-	+	-	+	+	+	+	+	+	+	+	-
HIS-Cdc20	-	+	-	-	-	+	+	-	-	+	+	-	+	+	+
BubR1	-	-	+	-	-	+	-	+	-	+	+	+	-	+	+
Mad2	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+
GST-Cdc20															
HIS-Cdc20															
BubR1															
Mad2															

Figure 44. The amino terminal of BubR1 is a potent inhibitor of Cdc20 in the presence of Mad2.

Cdc20 was incubated with increasing concentrations of BuBR1¹⁻³⁶³ in the (red triangles) absence or (blue squares) presence of Mad2. The resultant APC/C ubiquitination was assessed and quantified.



CHAPTER 5.

Conclusions and Future Directions

Conclusions and Implications:

Since the initial implication that unattached kinetochores may play a role in the activation and sustenance of the mitotic checkpoint, there have been many proposals as to what that role might be. In this body of work, we shed light upon the function of unattached kinetochores in conveying a checkpoint active state prior to the attachment of all chromosomes to the mitotic spindle. We demonstrate biochemically that unattached kinetochores amplify the production of a wait-anaphase inhibitor which restrains APC/C^{Cdc20} ubiquitination activity (Figures 13, 14, 15). By acting directly upon Mad2, kinetochores catalyze its activation and likely convert it into a conformer that can more readily bind to and inhibit Cdc20 (Figures 15, 21, 29). The recruitment of Mad2 to kinetochores is crucial for this process: hindrance of Mad2 recruitment to kinetochores by Mad1 antibody treatment (Figure 27) or in the case of Mad2 dimerization mutants (Figure 23) negates the function of unattached kinetochores in catalyzing an APC/C inhibitor (Figure 25, 26, 28). Thus this work also directly tests and supports the Mad2 template model of Mad2 activation (Figure 3, 22-28).

Although kinetochores act predominantly on Mad2 alone, both Mad2 and BubR1 are required for the inhibition of APC/C; complete APC/C inhibition cannot be sustained without one or the other (Figure 15). This mutual requirement manifests itself as synergy between Mad2 and BubR1 (Figure 12, 15). Downstream of Mad2 activation (Figure 29), unattached kinetochores promote the association of BubR1 and Bub3 to APC/C^{Cdc20} (Figure 37-40). This association is mediated by dimerization competent Mad2, though its own overall binding to Cdc20 is not equivalently increased (Figure 37-40). The eventual APC/C inhibitor consists mostly of BubR1 and Bub3 bound to Cdc20, with very little Mad2 bound within the same complex (Figure 41). The inhibitor produced by kinetochores is soluble (Figure 33), and directly binds the APC/C^{Cdc20} complex without need for Cdc20 sequestration (Figure 35). Thus we propose that unattached kinetochores act as catalytic platforms during prometaphase to catalyze the dimerization and structural activation of Mad2. The closed Mad2 conformation more readily binds to Cdc20, producing an initial Mad2-Cdc20 complex more readily accessible for BubR1 binding. Once BubR1 binds to Cdc20, APC/C is more potently inhibited, and Mad2 can dissociate from the inhibited complex. This frees up Mad2 to continue cycling onto unattached kinetochores, where it may act as a sensor for the kinetochore attachment state (Figure 42).

Surprisingly, BubR1 binds to free Cdc20 in the absence of Mad2 and unattached kinetochores (Figure 36), even though its binding to APC/C^{Cdc20} is

dependent upon both (Figures 37, 38, 40). The understanding that there are two distinct Cdc20 binding modules within BubR1 that may be differentially regulated (Davenport et al., 2006) raises the possibility that there may also be several pathways that converge upon BubR1 to inhibit premature APC/C^{Cdc20} activation. The checkpoint dependent pathway is likely to rely upon Mad2 to mediate the interaction between APC/C^{Cdc20} and BubR1 (Figure 44), and thus may have the potential to monitor and control that interaction based on kinetochore-microtubule attachment. The other, potentially non-Mad2 controlled pathway may represent a kinetochore-independent regulatory mechanism which could be responsible for inhibiting APC/C during the G2 period of interphase, as a cell prepares to enter mitosis. It may represent a pre-made inhibitor prior to mitotic entry to restrain APC/C activity before unattached kinetochore signaling has resumed. It provides an explanation for why MCC can be found in interphase cells (Sudakin et al., 2001) or why homologous complexes in yeast can be found in the absence of a functional kinetochore (Fraschini et al., 2001). It also provides an explanation for so-called mitotic timers (Meraldi et al., 2004). If there are pre-made inhibitors prior to mitotic entry, anaphase onset can be delayed temporarily by these inhibitors regardless of checkpoint function or unattached kinetochore signaling. The two differentially regulated pathways of Cdc20 inhibition by BubR1 present an exciting possibility which will be further explored in collaboration with Dr. Joo Seok Han.

Unresolved Questions and Future Directions:

The *in vitro* reconstitution of unattached kinetochore signaling demonstrated that unattached kinetochores catalyze the production of a wait anaphase inhibitor by acting on Mad2 and promoting the binding of BubR1 to APC/C^{Cdc20}. They provided evidence for a soluble inhibitor which can diffuse to bind and assemble upon a pre-activated APC/C. This system also opens the door to test many other crucial, yet lingering, questions that revolve around the mitotic checkpoint.

A significant question still remains about whether unattached kinetochores are solely a source of Mad1 which can recruit and activate Mad2 through the formation of Mad2 dimers. It is also unknown whether Mad1 at the kinetochores differs from free Mad1 that may be found in the mitotic cytosol. Are there additional components on kinetochores that play a role in the production of an anaphase inhibitor? Purified chromosomes can be compared to purified Mad1, either free or bead bound, in inhibiting APC/C ubiquitination activity.

Additional checkpoint proteins such as CenpE and Bub1 are stably localized to unattached kinetochores (Figure 7) and are essential for the metazoan mitotic checkpoint (Musacchio and Salmon, 2007). It is unclear if they have a direct role in the production of an APC/C inhibitor, though Bub1 has been implicated in the sensitization of Cdc20 to checkpoint inhibition (Tang et al., 2004). Such questions can now be tested. Instead of the addition

of purified versions of these proteins, chromosomes can be isolated from cells in which these components have been depleted, and tested for competency in magnifying APC/C inhibitor production.

Phosphorylation of a number of substrates in mitosis is crucial for the promotion and maintenance of a mitotic state. There are a number of kinases which play a role in the mitotic checkpoint such as Mps1, Plk1, Aurora, TAO1 and PICH (reviewed in Chapter 1). Additionally, both Bub1 and BubR1 have kinase activities which are required for the checkpoint (Basu et al., 1999; Chan et al., 1999; Taylor and McKeon, 1997), though it is unclear to what degree, if any, those kinase activities contribute to APC/C inhibition. The overall kinase requirement in APC/C inhibitor production can be tested by the addition and/or quenching of ATP in ubiquitination assays, while the contribution of each individual kinase can be tested by the addition of purified protein or the depletion from kinetochores of stably bound kinases.

In vitro reconstitution of unattached kinetochore signaling allows us not only to test not only how a wait anaphase inhibitor can be produced, but also how it can be turned off. P31/Comet is an antagonist of the checkpoint (Xia et al., 2004). It binds to Mad1 or Cdc20 bound Mad2 (Luo et al., 2004) in a manner that is mutually exclusive to dimer formation (Mapelli et al., 2006; Yang et al., 2007), but how it functions to turn off kinetochore mediated Mad2 signaling is not well understood. How p31 is regulated so that it does not prematurely inhibit Mad2 function is not known either. Addition of purified p31

to *in vitro* assays reconstructing kinetochore and Mad2 signaling can shed light upon these questions as well.

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