Developmental Cell *16*

Supplemental Data

Unattached Kinetochores Catalyze Production of an Anaphase Inhibitor that Requires a Mad2 Template to Prime Cdc20 for BubR1 Binding

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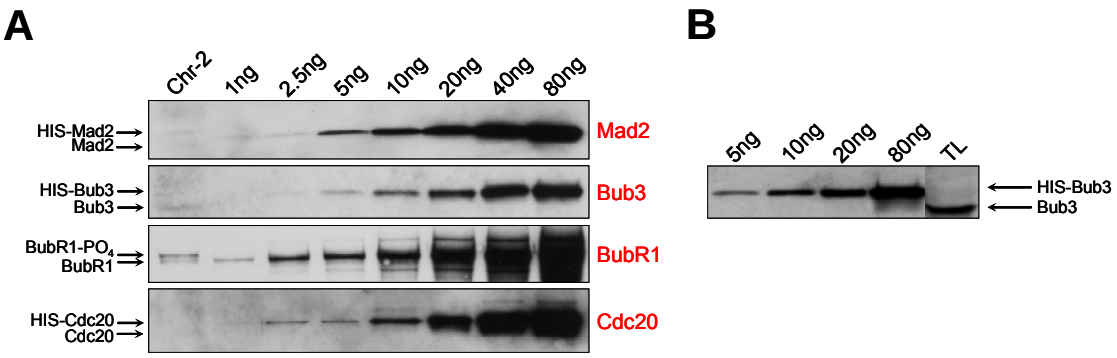

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	Estimated Cellular Concentration (nM)	Molecules per cell	Molecules remaining on chromosomes per cell volume	Percentage of Initial Cellular Concentration
Mad2	120	66,000	<710	<1.1%
Bub3	195	107,000	1000	0.9%
BubR1	90	50,000	220	0.4%
Cdc20	100	55,000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

Figure S1. 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 x 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 x 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).

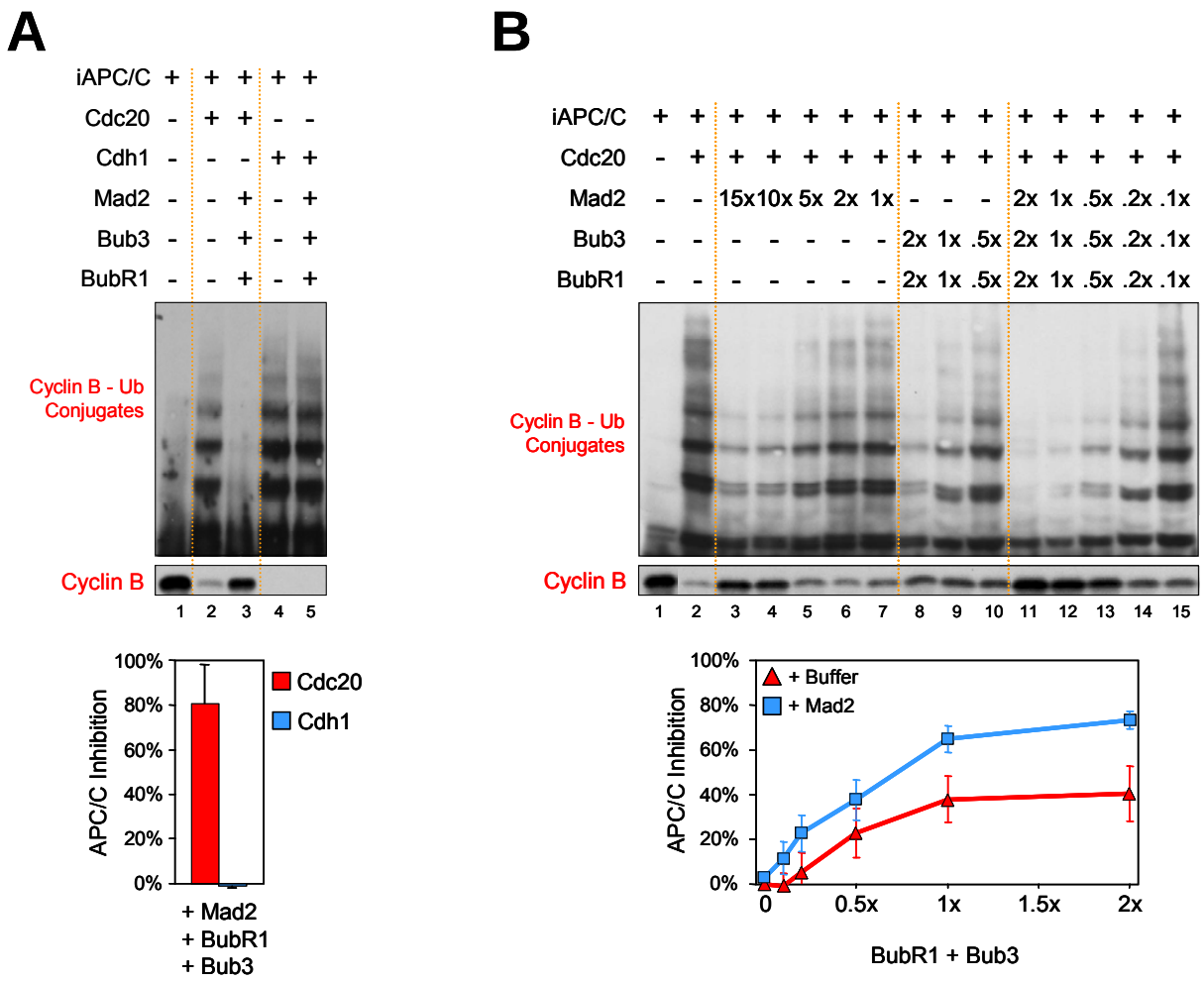


Figure S2. Synergy of APC/C^{Cdc20} Inhibition by Addition of Mad2 to Bub3/BubR1

(A) Specificity of inhibition by Mad2, BubR1 and Bub3 for blocking activation of APC/C^{Cdc20} was tested by the comparison of (red) Cdc20 versus (blue) 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), the quantification of which is depicted below.

(B) Synergy of inhibition of APC/C^{Cdc20} from addition of (red) Mad2 to Bub3/BubR1 and Cdc20 prior to APC/C addition and activity measurement compared to corresponding APC/C activity (blue) without Mad2 addition. APC/C activity was assayed as in (A).

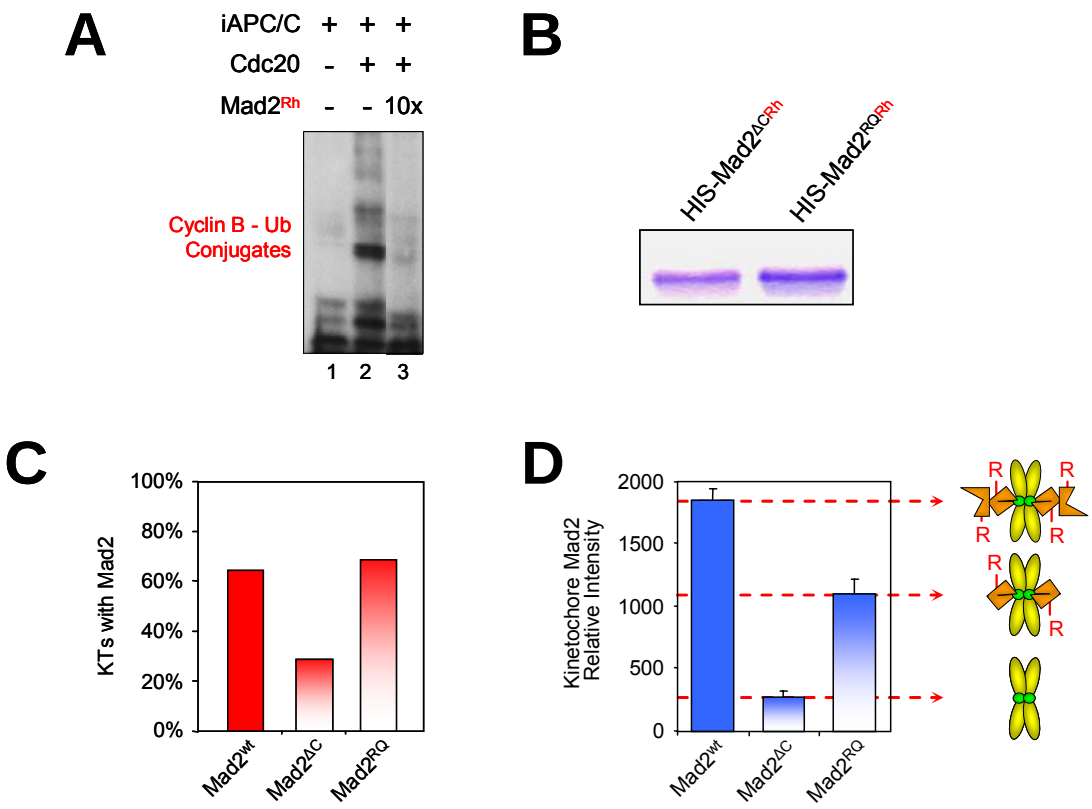


Figure S3. Kinetochores on Purified Chromosome Amplify Inhibition of Mitotic APC/C^{Cdc20}

(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. APC/C activity was quantified by the presence of lower mobility ubiquitin-conjugated cyclin B species.

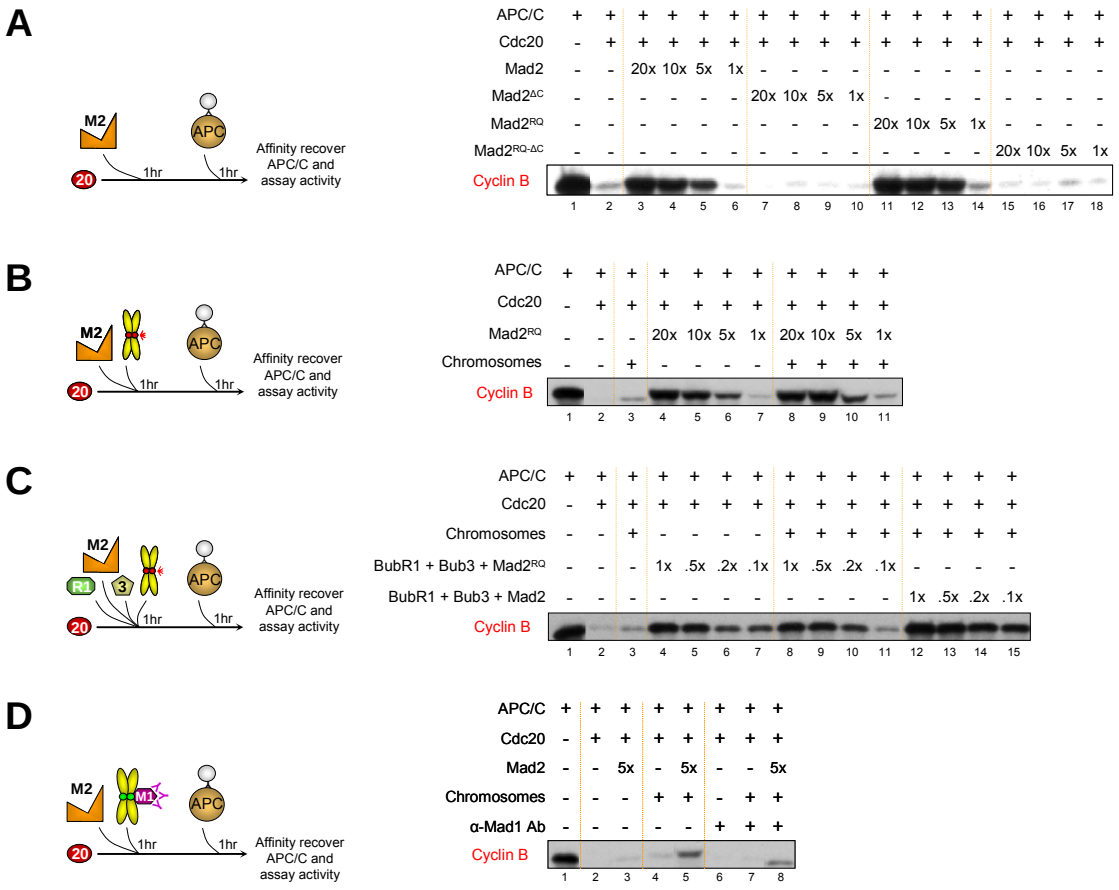
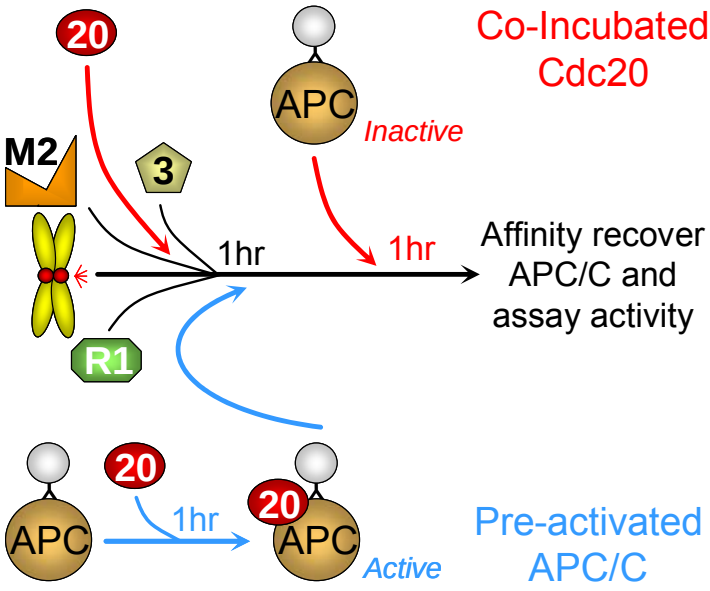


Figure S4. Recruitment of Mad2 (Wild-Type or Dimerization Incompetent) to Unattached Kinetochores Containing Already Bound Mad1

(A) Rhodamine labeled recombinant Mad2 retains ability to inhibit Cdc20 activation of APC/C, as assessed by the presence of ubiquitin conjugated cyclin B species.

(B) Mad1 binding deficient Mad2$^{\Delta C}$ and dimerization incompetent Mad2^{RQ} after purification and covalent conjugation to rhodamine visualized after Coomassie staining.

(C and D) Purified chromosomes were incubated with rhodamine-labeled Mad2^{wt}, Mad2$^{\Delta C}$, 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$^{\Delta C}$, 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$^{\Delta C}$ binding was near background levels.



APC/C	+	+	+	+	+	+	+	+	+	+	+	+
Cdc20	-	+	+	+	+	+	-	+	+	+	+	+
Mad2 + BubR1 + Bub3	-	-	+	+	+	+	-	-	+	+	+	+
Chromosomes	-	-	+	+	+	+	-	-	+	+	+	+

Figure S5. Mad2 Conformational Change and Dimerization Is Required for Kinetochore-Dependent Amplification of a Cdc20 Inhibitor
APC/C inhibition assays for $Mad2^{wt}$ and dimerization incompetent $Mad2^{RQ}$ with or without added Bub3/BubR1. Cyclin B ubiquitination assays corresponding to the data quantified in (A) Figure 3D, (B) Figure 3E, and (C) Figure 3F.

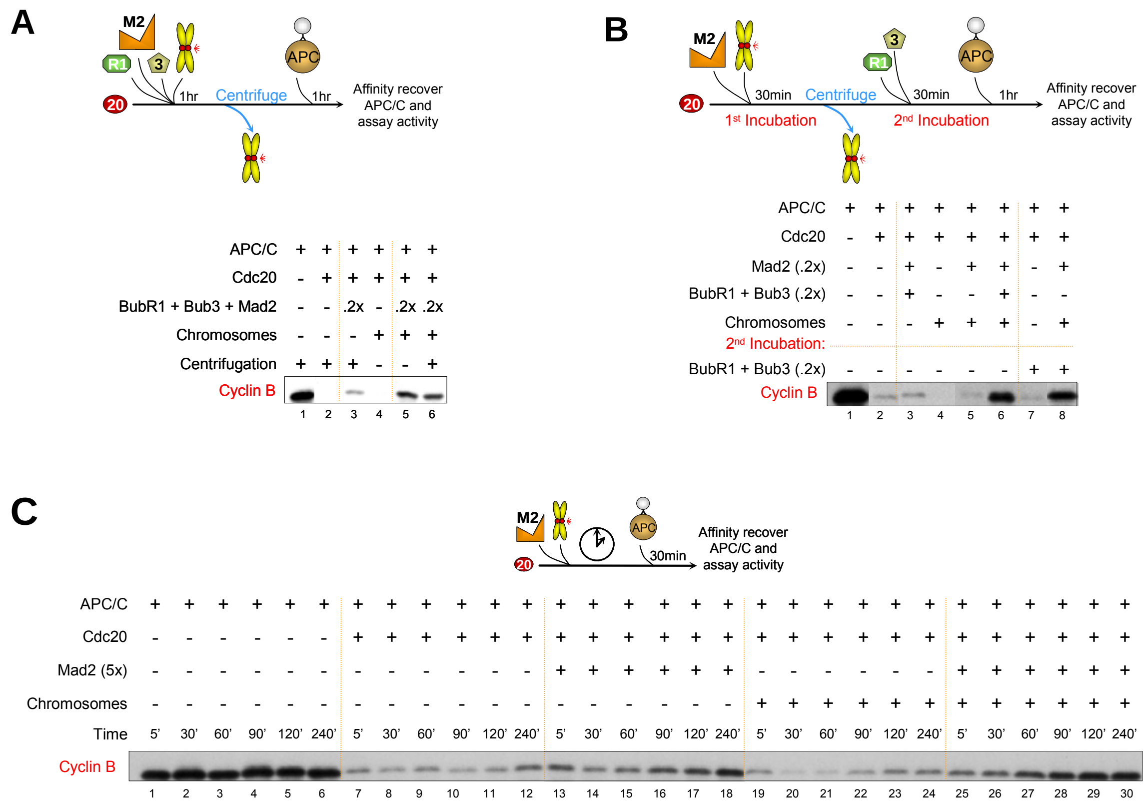


Figure S6. Chromosomal Amplification Cdc20-Stimulated APC/C Inhibition
(A) Purified chromosomes were incubated with Mad1 antibody prior to addition of a five-fold excess of Mad2 to Cdc20, and finally addition to APC/C ubiquitination assays.
(B) Kinetochores facilitate BubR1, Bub3, and Mad2 association with APC/C. Checkpoint components were co-incubated together with Cdc20 and chromosomes, followed by APC/C addition and assay for its activity. 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) Kinetochores produce a diffusible Cdc20 inhibitor. Chromosomes were incubated with BubR1, Bub3, Mad2, and Cdc20 (1:1:1:5); the chromosomes were subsequently removed by centrifugation, and the supernatant fraction was assayed for activation of APC/C for cyclin B ubiquitination. Parallel assay was done without chromosome removal or without initial chromosome addition.