# BubR1 and APC/EB1 cooperate to maintain metaphase chromosome alignment

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The accurate segregation of chromosomes in mitosis requires the stable attachment of microtubules to kinetochores. The details of this complex and dynamic process are poorly understood. In this study, we report the interaction of a kinetochore-associated mitotic checkpoint kinase, BubR1, with two microtubule plus end–associated proteins, adenomatous polyposis coli (APC) and EB1, providing a potential link in stable kinetochore microtubule attachment. Using immunodepletion from and antibody addition to *Xenopus laevis* egg extracts, we show that BubR1 and its kinase activity are essential for positioning chromosomes at the metaphase plate. BubR1 associates with APC and EB1 in egg extracts, and the complex formation is necessary for metaphase chromosome alignment. Using purified components, BubR1 directly phosphorylates APC and forms a ternary complex with APC and microtubules. These findings support a model in which BubR1 kinase may directly regulate APC function involved in stable kinetochore microtubule attachment.

## Introduction

The challenge of mitosis is to faithfully transmit chromosomes in each cell division. Errors in this process cause aneuploidy (abnormal numbers of chromosomes), which is frequently found in cancers and is believed to promote the growth and progression of diseases (Hartwell and Kastan, 1994). Accurate chromosome segregation requires proper mitotic spindle formation and successful chromosome movement along the spindle. Chromosomes capture spindle microtubules through a dynamic search and capture mechanism by their kinetochores. Numerous proteins, including motors and nonmotor proteins, have been implicated in stable kinetochore microtubule attachment, although mechanistically, the role for the majority of these proteins has yet to be identified (Biggins and Walczak, 2003; Cleveland et al., 2003; Kline-Smith et al., 2005).

A group of microtubule-associated proteins has emerged to participate in kinetochore microtubule attachment. Adenomatous polyposis coli (APC) and its binding partner EB1 (Su et al., 1995) localize to kinetochores during mitosis in a microtubule-dependent manner (Fodde et al., 2001; Kaplan et al., 2001). Defects in spindle formation and chromosome missegregation have been shown in mammalian cultured cells harboring a colon cancer–related dominant APC mutant (Green and Kaplan, 2003; Tighe et al., 2004). APC and EB1 depletion has also been reported to give rise to similar defects in chromosome congression without arresting cells in mitosis based on a fixed cell characterization (Kaplan et al., 2001; Green et al., 2005). Using a combination of RNAi-mediated protein depletion and live cell imaging, it has been shown that chromosomes in APC- or EB1-depleted cells do congress to the spindle equator, although APC-depleted cells exhibited transient mitotic checkpoint–dependent anaphase delay (Draviam et al., 2006). The depletion of APC from cytostatic factor (CSF)–arrested *Xenopus laevis* egg extracts has been shown to cause a decrease in spindle microtubule density (Dikovskaya et al., 2004), although it is not well understood how bipolar spindles are formed in noncycled egg extracts with sperm nuclei (haploid). Spindles formed in cycled egg extracts were not sensitive to APC depletion (Dikovskaya et al., 2004). APC associates with the microtubule-destabilizing protein mitotic centromere-associated kinesin (MCAK) in *Xenopus* egg extracts (Banks and Heald, 2004). Codepletion of APC and MCAK from cycled egg extracts results in large, dense microtubule structures surrounding the chromosomes (Banks and Heald, 2004). Furthermore, APC has been shown to form a complex with mitotic checkpoint proteins Bub1 and Bub3 and is a substrate for Bub1/BubR1 kinases in vitro (Kaplan et al., 2001); however, the physiological role of the APC–Bub1/BubR1 interaction is not known.

The mitotic checkpoint (spindle assembly checkpoint) inhibits premature anaphase onset until every chromosome has successfully attached to microtubules. Mitotic kinases BubR1 (Chan et al., 1999; Chen, 2002; Mao et al., 2003) and Bub1 (Taylor and McKeon, 1997) are essential for the metazoan mitotic

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Abbreviations used in this paper: APC, adenomatous polyposis coli; CENP, centromere protein; CSF, cytostatic factor; KD, kinase dead; MCAK, mitotic centromere-associated kinesin; NTA, nitrilotriacetic acid; WT, wild type.


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checkpoint. Recent studies have suggested that BubR1 (Lampson and Kapoor, 2005) and Bub1 (Johnson et al., 2004; Meraldi and Sorger, 2005) are also necessary for the stabilization of kinetochore microtubule capture; however, it is not known whether their actions are direct or not and whether the kinase activity is required for this. Taking advantage of *Xenopus* egg extracts that are naturally arrested in metaphase of meiosis II by CSF, we report here that the complex formation between BubR1 and two microtubule plus end–interacting proteins, APC and EB1, is essential for chromosome alignment at the metaphase plate, providing a potential link in stable kinetochore microtubule attachment.

## Results

### BubR1 and its kinase activity are required for metaphase chromosome alignment

To investigate the role of BubR1 in kinetochore microtubule attachment and metaphase chromosome alignment, we first perturbed BubR1 function in *Xenopus* egg extracts by the addition of a specific BubR1 antibody, which inhibits BubR1 kinase activity and the mitotic checkpoint (Mao et al., 2003). *Xenopus* egg extracts were cycled through interphase to generate duplicated sister chromatids and were arrested at the subsequent M phase upon the addition of an aliquot of egg extracts containing both active Cdk1 and CSF. Although the addition of a control IgY antibody to egg extracts yielded predominantly bipolar spindles with chromosomes aligned at the metaphase plate (Fig. 1, A and C), the addition of an anti-BubR1 antibody produced normal bipolar spindles with misaligned chromosomes (Fig. 1, B and C). Prolonged incubation failed to produce bipolar spindles with properly aligned chromosomes. Similar results were obtained in three independent experiments. Addition of the BubR1 antibody does not prevent the kinetochore binding of BubR1 or its binding partner centromere protein E (CENP-E; Mao et al., 2003). This clearly indicates that BubR1 and its kinase activity are essential for chromosome alignment.

To further test the requirement for BubR1 and its kinase activity in chromosome alignment, BubR1 was quantitatively immunodepleted from egg extracts (Fig. 1 K). Mock-depleted egg extracts produced bipolar spindles with chromosomes congressed at the metaphase plate (Fig. 1, D and J); BubR1-depleted egg extracts generated bipolar spindles with severe chromosome alignment defects (Fig. 1, E and J). Purified recombinant wild-type (WT) BubR1 or a kinase-inactive point mutant (kinase-dead [KD] BubR1; Fig. 1 L) efficiently bound to kinetochores when added to depleted egg extracts (Mao et al., 2003). The addition of recombinant kinase-competent BubR1 (Fig. 1, H and J) but not the KD-BubR1 mutant (Fig. 1, I and J) to a level comparable with that of endogenous BubR1 completely restored metaphase chromosome alignment. Although CENP-E kinetochore association is BubR1 dependent in *Xenopus* egg extracts, kinetochore-bound KD-BubR1 is able to stimulate the binding of a normal level of CENP-E (Mao et al., 2003).

As an additional test of stable kinetochore microtubule attachment, we treated egg extracts with a low dose of nocodazole (100 nM), which causes a preferential disassembly of astral microtubules (O'Connell and Wang, 2000). In control egg extracts, the mitotic spindles remained intact 10 min after nocodazole treatment, although the spindle length was reduced (Fig. 1 F). In BubR1-depleted egg extracts, however, only a few microtubules were observed (Fig. 1 G). Altogether, these findings support a model in which BubR1 and its kinase activity are essential for positioning chromosomes at the metaphase plate in *Xenopus* egg extracts.

### BubR1 interacts with APC/EB1 in *Xenopus* egg extracts

To investigate potential binding partners of BubR1 that could account for its function in chromosome alignment, we performed coimmunoprecipitation experiments with a specific anti-BubR1 antibody in *Xenopus* egg extracts with the addition of sperm nuclei. In BubR1 immunoprecipitates, besides the known binding partner CENP-E (Fig. 2 A, CENP-E in lane 4), immunoblotting analysis also identified two microtubule-associated proteins, APC and EB1, as binding partners of BubR1 (Fig. 2 A, APC and EB1 in lane 4). To verify the interaction between APC/EB1 and BubR1, APC was quantitatively immunoprecipitated from egg extracts (Fig. 2 B, APC in lane 4) with a specific APC antibody (Fig. 2 F). EB1 was detected in the immunoprecipitates (Fig. 2 B, EB1 in lane 4) as expected. Furthermore, a portion of endogenous BubR1 was also found in the immunoprecipitates (Fig. 2 B, BubR1 in lane 4), suggesting that BubR1 and APC/EB1 could exist in a complex. Also, a portion of BubR1 was cofractionated with APC and EB1 after sucrose density gradient centrifugation (Fig. 2 C), supporting the conclusion that these proteins form a physical complex.

To address whether APC works together with BubR1 to regulate kinetochore microtubule capture, we first examined their localization in cycled egg extracts. *Xenopus* egg extracts were cycled through interphase to allow DNA and kinetochore replication and then were cycled back into mitosis. As shown in Fig. 2 D, APC was detected along spindle microtubules and colocalized with BubR1 at attached kinetochores.

If the BubR1–APC/EB1 interaction is required for stable kinetochore microtubule attachment, the complex formation of BubR1–APC/EB1 should be enhanced when microtubules are attached to kinetochores. To test this possibility, we conducted APC immunoprecipitation analysis in CSF-arrested *Xenopus* egg extracts with or without added sperm nuclei (kinetochores) and nocodazole (to disassemble microtubules; as indicated in Fig. 2 E). Immunoprecipitates obtained with an APC antibody were found to contain comparable levels of APC and EB1 under all conditions (Fig. 2 E, APC and EB1 in lanes 1–3), suggesting that the mitotic APC/EB1 interaction is independent of kinetochores and spindle microtubules. In contrast, there was considerably greater amounts of BubR1 ($\sim$50% increase) in APC precipitates from egg extracts containing spindle structures and attached kinetochores (Fig. 2 E, BubR1 in lane 2) than that from control CSF-arrested egg extracts (Fig. 2 E, BubR1 in lane 1) or egg extracts with unattached kinetochores (in the presence of nocodazole; Fig. 2 E, BubR1 in lane 3). This strongly suggests that complex formation between BubR1 and APC/EB1 is enhanced in the presence of spindle microtubules and attached kinetochores.

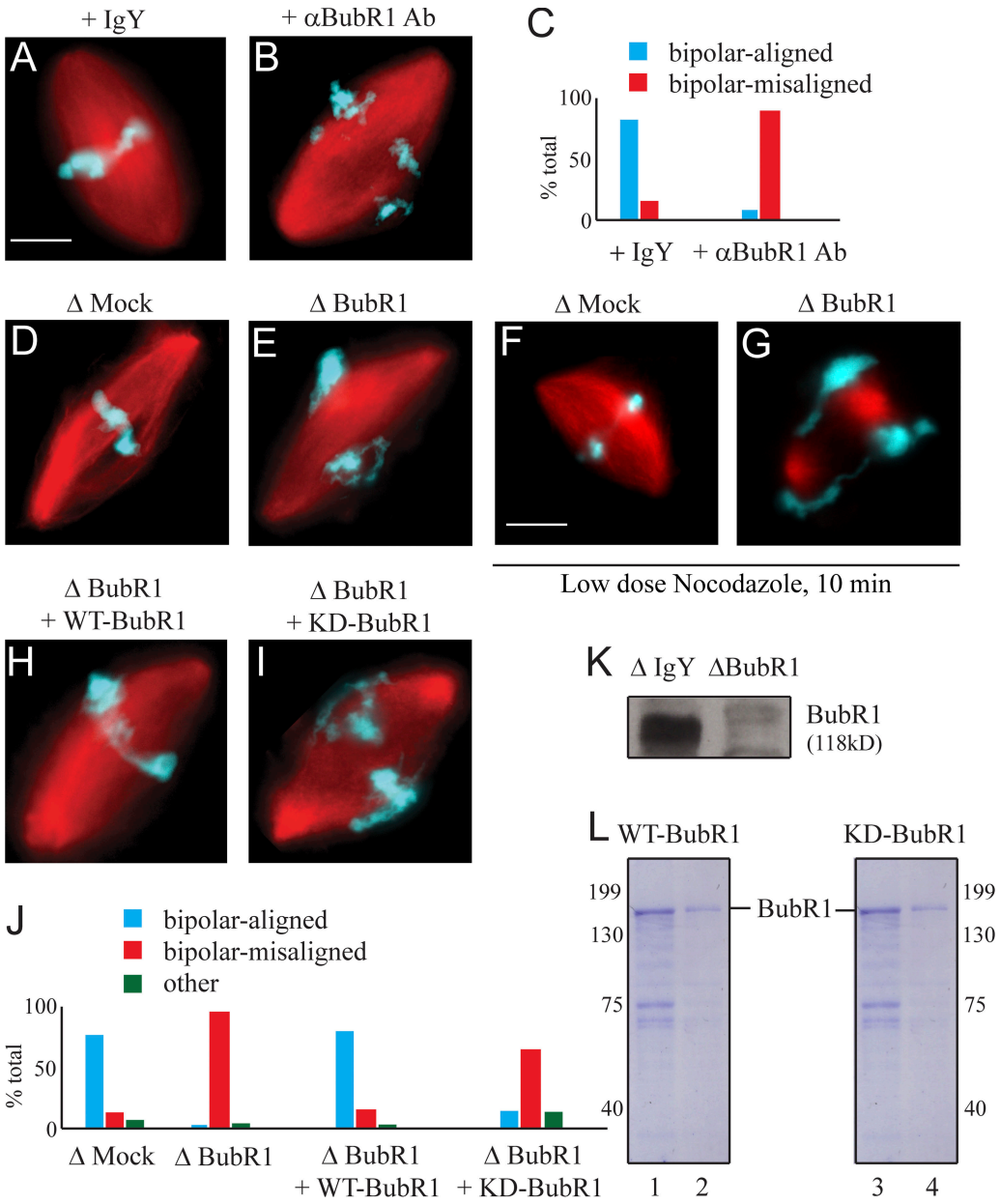


Figure 1. **BubR1 and its kinase activity are essential for metaphase chromosome alignment in *Xenopus* egg extracts.** (A, B, D, E, H, and I) Representative mitotic structures formed in the presence of IgY (A) and BubR1 antibody (B) or mock depleted (D), BubR1 depleted (E), and BubR1 depleted and supplemented with recombinant WT-BubR1 (H) or KD-BubR1 (I). (C and J) Quantification of structures formed from sperm nuclei in cycled egg extracts as indicated scored 80 min after exit from interphase. At least 50 mitotic structures were scored for each extract. Data are presented from one representative experiment. (F and G) Mitotic structures formed in egg extracts treated with a low dose of nocodazole (100 nM) for 10 min. (K) CSF egg extracts were immunodepleted of BubR1 using purified IgY or BubR1 antibodies, and BubR1 protein was assayed by immunoblotting. (L) Purification of recombinant *Xenopus* WT- and KD-BubR1. Initial *E. coli* lysates encoding GST–WT-BubR1 (lane 1) and GST–KD-BubR1 (lane 3) and GST–WT-BubR1 (lane 2) and GST–KD-BubR1 (lane 4) after affinity purification over immobilized glutathione. Bars, 10 μm.

### APC and EB1 are essential for chromosome alignment in *Xenopus* egg extracts

We have demonstrated that BubR1 and its kinase activity are necessary for metaphase chromosome alignment (Fig. 1). Because BubR1 cannot directly bind to microtubules, it is more likely that BubR1 regulates the activity of a microtubule-binding protein. Thus, we examined the effects of APC and EB1 immunodepletion on metaphase chromosome alignment in *Xenopus* egg extracts. The depletion of either APC or EB1 with specific antibodies (Fig. 3, H and I) produced bipolar spindles with misaligned chromosomes (Fig. 3, B, D, and G). Again, extended incubation failed to produce bipolar spindles with properly aligned chromosomes.

To confirm that the chromosome alignment defect is caused by the loss of APC or EB1 rather than other associated proteins, we reconstituted APC or EB1 in APC- or EB1-depleted egg extracts with purified recombinant APC (Fig. 3 J) or EB1 (Fig. 3 K) proteins to approximately the normal level, and proper metaphase chromosome alignment was restored (Fig. 3, C, E, and G). On the basis of this apparently complete reconstitution,

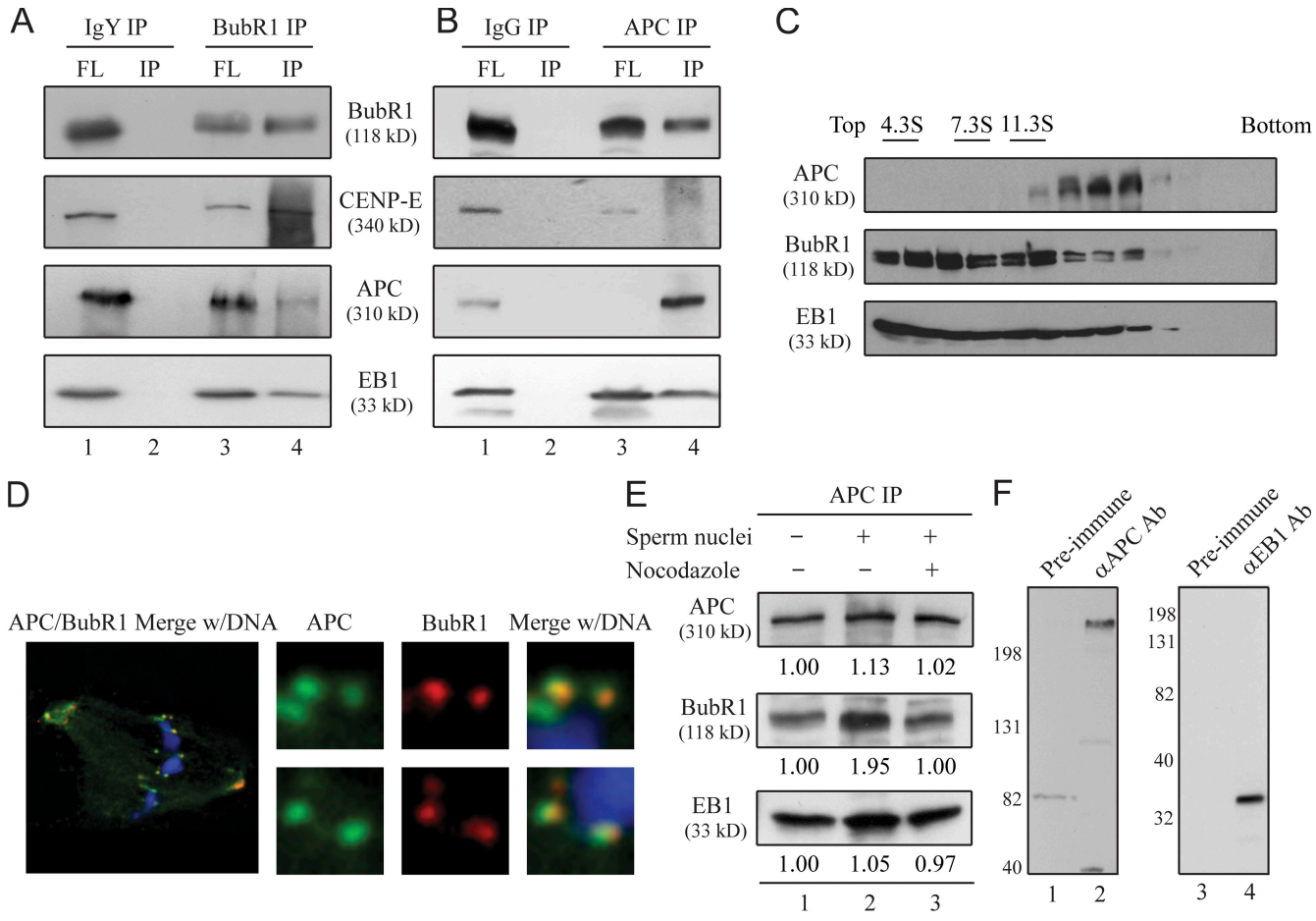


Figure 2. **BubR1 forms a complex with APC/EB1 in *Xenopus* egg extracts.** (A and B) Immunoprecipitates from CSF-arrested egg extracts containing sperm nuclei with affinity-purified chicken anti-BubR1 IgY (A) or rabbit anti-APC IgG (B) antibodies probed with BubR1, CENP-E, APC, or EB1 antibodies. FL, extracts after immunoprecipitation; IP, immunoprecipitates. A threefold higher proportion of the bead-bound fraction relative to the depleted extracts was analyzed. (C) Cofractionation of BubR1 with APC and EB1 after sucrose density gradient centrifugation. The high speed supernatant of metaphase egg extracts was separated over a 5–15% continuous sucrose gradient. Fractions were collected and analyzed by immunoblotting with the indicated antibodies. (D) Indirect immunofluorescence image of APC (green), BubR1 (red), and DNA (blue) in cycled egg extracts. APC and BubR1 are shown magnified in the insets. (E) Immunoprecipitates from CSF-arrested egg extracts containing or not containing sperm nuclei and nocodazole (as indicated) with affinity-purified rabbit anti-APC IgG probed with APC (top), BubR1 (middle), or EB1 (bottom) antibodies. Immunoreactivity was quantified relative to the value in lane 1. (F) Characterization of APC and EB1 antibodies. CSF-arrested egg extracts were immunoblotted with preimmune (lanes 1 and 3) or anti-APC or -EB1 antibodies (lanes 2 and 4).

we believe that the disruption of chromosome alignment in APC- or EB1-depleted egg extracts truly reflects APC or EB1 depletion and not the depletion of some associated partners. Codepletion of APC and EB1 from *Xenopus* egg extracts produced similar chromosome alignment defects as APC or EB1 single depletion (Fig. 3, F and G). Furthermore, a majority of spindle microtubules in APC- or EB1-depleted egg extracts was not stable in the presence of a low dose of nocodazole (Fig. 3, M and N). These results demonstrate that the microtubule-associated proteins APC and EB1 are necessary for metaphase chromosome alignment.

### Depletion of APC and EB1 arrests *Xenopus* egg extracts in mitosis

Because BubR1 is an essential mitotic checkpoint kinase, we decided to examine whether its binding partners APC and EB1 are also necessary for mitotic checkpoint signaling in *Xenopus* egg extracts. Sperm nuclei and the microtubule assembly inhibitor nocodazole were added to APC- or EB1-depleted egg extracts to assemble condensed mitotic chromosomes and produce unattached kinetochores. In mock-depleted egg extracts, this yielded an activated mitotic checkpoint as judged by the absence of nuclear envelope reassembly, a continuous high level of Cdc2 kinase activity after the calcium-mediated inactivation of CSF (Fig. 4 A, blot b), and kinetochore recruitment of BubR1 and Mad2 (Fig. 4 C). After BubR1 depletion, no BubR1 and Mad2 could be detected at unattached kinetochores (Fig. 4 C), and the mitotic checkpoint was not activated (Fig. 4 A, blot c) as expected (Chen, 2002; Mao et al., 2003). The depletion of APC or EB1 neither prevented the kinetochore association of BubR1 and Mad2 (Fig. 4 C) nor abrogated the mitotic checkpoint response in the presence of nocodazole (Fig. 4 A, blots d and g). Furthermore, even after spindle assembly in the absence of nocodazole, APC- or EB1-depleted egg extracts produced chronic mitotic arrest, as revealed by continued Cdc2 kinase activity and condensed chromosomes (Fig. 4 A, blots e and h). On the other hand, APC- or EB1-depleted egg extracts supplemented with recombinant APC or EB1, respectively, showed no checkpoint arrest,

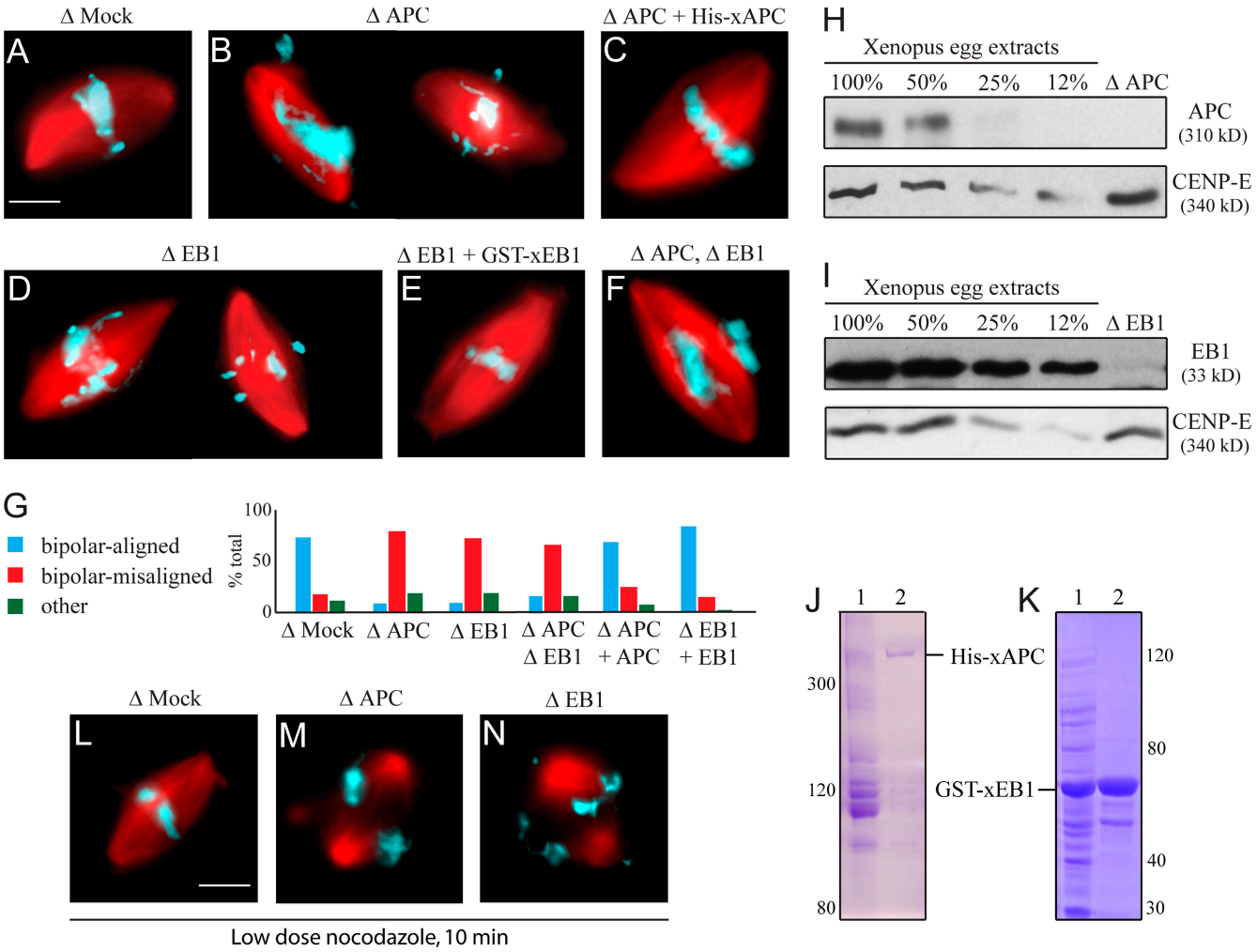


Figure 3. **APC and EB1 depletion results in chromosome alignment defects.** (A–F) Metaphase spindles assembled in mock- (A), APC- (B), EB1- (D), or APC/EB1 (F) -depleted egg extracts and APC- or EB1-depleted egg extracts supplemented with purified recombinant APC (C) or EB1 (E). (G) Quantification of structures formed from sperm nuclei in cycled egg extracts as indicated scored 80 min after exit from interphase. At least 50 mitotic structures were scored for each extract. Data are presented from one representative experiment. (H and I) Immunoblot of immunodepleted CSF egg extracts. CSF-arrested egg extracts were immunodepleted of APC or EB1 using APC and EB1 antibodies. 1 μl of control extracts with serial dilution (as indicated) or 1 μl of depleted extracts was analyzed for APC (H) or EB1 (I) levels. (J and K) Purification of recombinant *Xenopus* APC (J) and EB1 (K). (J) An initial whole cell extract from insect cells infects with a baculovirus encoding His-APC (lane 1) and purified APC (lane 2) after purification over immobilized Ni-NTA agarose. (K) An initial *E. coli* lysate encoding GST-EB1 (lane 1) and GST-EB1 after affinity purification over immobilized glutathione (lane 2). (L–N) Mitotic structures formed in egg extracts treated with a low dose of nocodazole (100 nM) for 10 min. Bars, 10 μm.

inactivating Cdc2 kinase and reassembling nuclei around decondensing chromosomes within 60 min (Fig. 4 A, blots f and i).

BubR1 kinase activity is essential for the mitotic checkpoint (Mao et al., 2003, 2005; Kops et al., 2004). After the depletion of endogenous BubR1 and the restoration of normal BubR1 levels with purified GST-tagged BubR1 (Mao et al., 2003, 2005), BubR1 kinase activity was measured by affinity recovery of BubR1 with GST antibody beads from CSF-arrested egg extracts. BubR1 kinase was activated only when microtubule assembly was inhibited (Fig. 4 B, lane 2) and was silenced, as expected, after spindle assembly and chromosome alignment (Fig. 4 B, lane 1). However, in APC- or EB1-depleted egg extracts, BubR1 kinase activity was undiminished even after spindle assembly (Fig. 4 B, lanes 4 and 6). Altogether, these results are consistent with the conclusion that APC and EB1 are necessary for proper kinetochore microtubule attachment.

## Complex formation of BubR1–APC/EB1 is essential for its function in chromosome alignment

In mammalian cultured cells, it has been shown that the BubR1 RNAi phenotype is partially rescued by an Aurora kinase inhibitor: many chromosomes aligned at the metaphase plate, with a few chromosomes remaining near the poles (Lampson and Kapoor, 2005), suggesting that BubR1 and Aurora B might regulate opposite activities. To directly test this hypothesis, we coimmunodepleted APC and Aurora B from egg extracts. In APC and Aurora B codepleted extracts, only ~50% of mitotic figures were bipolar spindles (Fig. 5 I), which is consistent with an earlier report that the chromosomal passenger complex is required for bipolar spindle formation in egg extracts (Sampath et al., 2004); however, the number of bipolar spindles with aligned chromosomes increased (Fig. 5, D and I). Codepletion of BubR1 and

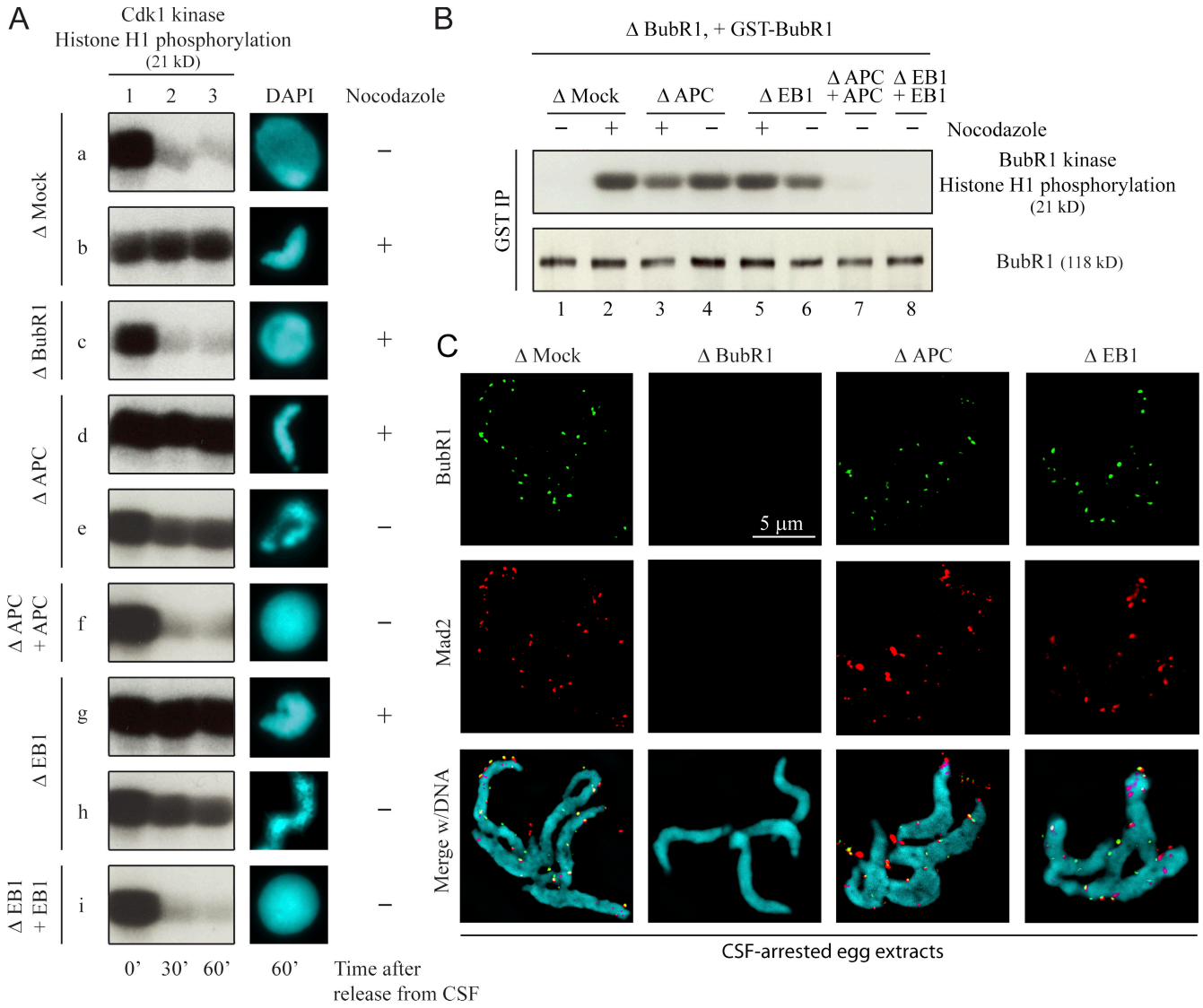


Figure 4. **APC/EB1 depletion arrests *Xenopus* egg extracts in mitosis.** (A) CSF-arrested *Xenopus* egg extracts were mock depleted (a and b), BubR1 (c), APC (d and e), or EB1 (g and h) depleted, or APC or EB1 depleted and supplemented with recombinant APC (f) and EB1 (i). After incubation of sperm nuclei with or without nocodazole (as indicated), CSF activity was inactivated by the addition of calcium. Aliquots were taken from each extract at the indicated times and assayed by autoradiography for Cdk1 kinase activity (left) using added histone H1 as a substrate and (right) maintenance of chromatin condensation. (B) The depletion of APC and EB1 activates BubR1 kinase in CSF-arrested *Xenopus* egg extracts. After immunodepletion of endogenous BubR1, recombinant GST-BubR1 was added to a molar level comparable with the endogenous level of BubR1 in CSF-arrested egg extracts. Sperm nuclei and nocodazole were then added to mock-, APC-, or EB1-depleted egg extracts or supplemented with recombinant APC and EB1 as indicated. After 30 min, GST-BubR1 was immunoprecipitated using specific anti-GST antibodies and immunoblotted with anti-BubR1 antibody (bottom) or kinase activity assayed after the addition of histone H1 and γ-[$^{32}$P]ATP (top). (C) CSF-arrested egg extracts were mock depleted or BubR1, APC, and EB1 depleted. After the addition of sperm nuclei and nocodazole, extracts were observed by indirect immunofluorescence with anti-BubR1 (green) and Mad2 (red) antibodies, and chromatin was visualized with DAPI (blue).

Aurora B has shown similar results (Fig. 5 E). In contrast, co-depletion of BubR1 and APC (and EB1) resulted in similar chromosome alignment defects as BubR1 or APC depletion alone (Fig. 5, B, C, and I). Furthermore, the spindles formed in Aurora B–depleted egg extracts were shorter, probably as a result of the increased activity of MCAK because Aurora B can phosphorylate MCAK and reduce its microtubule-depolymerizing activity in vitro (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). Indeed, egg extracts coimmunodepleted of APC and MCAK produced large microtubule structures surrounding the chromosomes instead of bipolar spindles (Fig. 5 F), which is consistent with an earlier study using an APC antibody depleted of both APC and MCAK from egg extracts (Banks and Heald, 2004).

It has been shown that an N-terminal fragment (APC$^{1–1,450}$ or N-APC) similar to the mutant protein expressed in many colorectal cancers dominantly compromises microtubule attachment in mitosis in mammalian cultured cells, resulting in errors in chromosome segregation (Green and Kaplan, 2003). We have expressed and purified the corresponding *Xenopus* truncated form of APC from *Escherichia coli* (Fig. 5 J, lane 2) and have tested its effect on metaphase chromosome alignment. In contrast to the GST control, mitotic spindles that

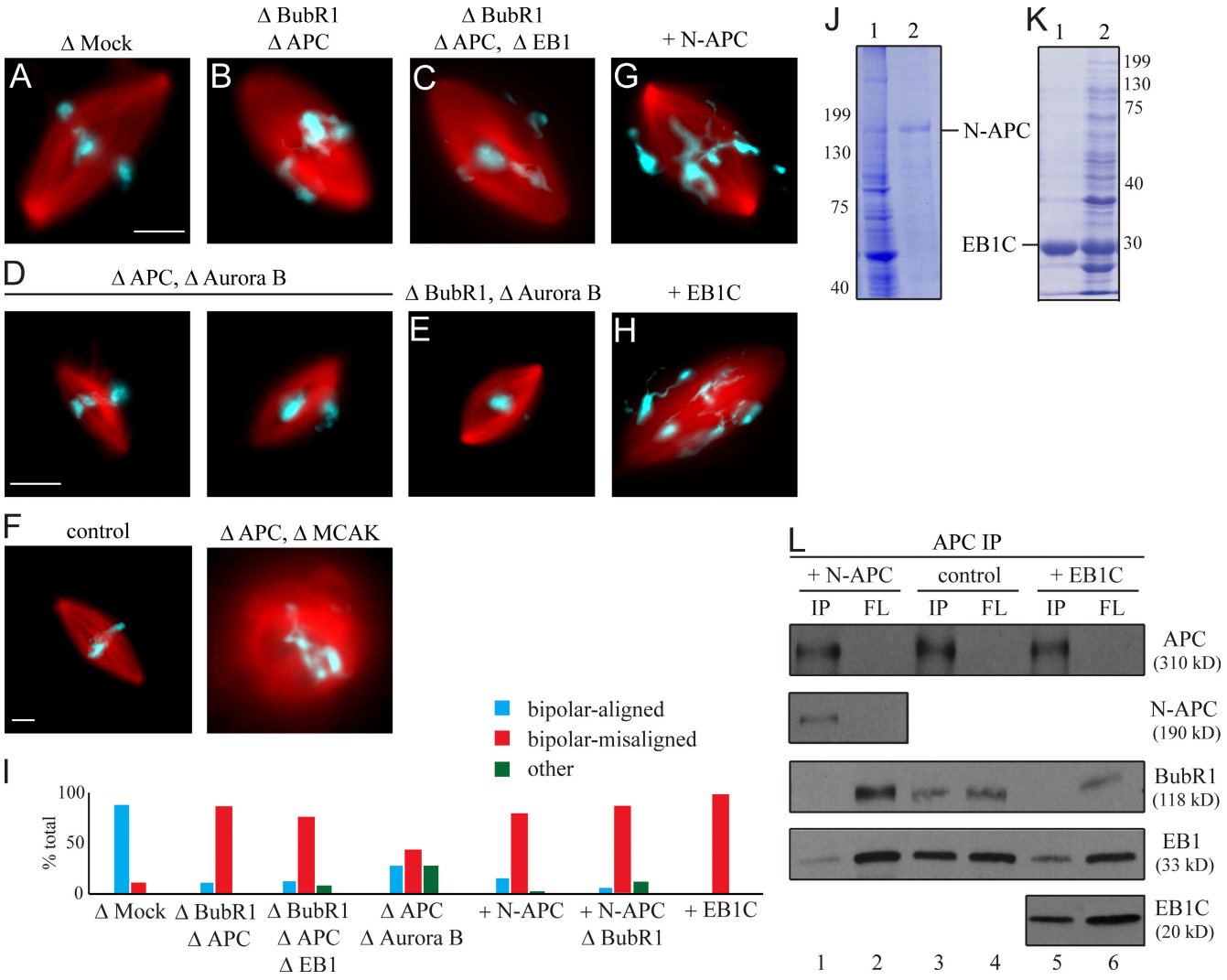


Figure 5. **BubR1–APC/EB1 complex formation is important for metaphase chromosome alignment.** (A–H) Metaphase spindle structures assembled in mock- (A and E), BubR1/APC- (B), BubR1–APC/EB1- (C), APC/Aurora B- (D), BubR1/Aurora B- (E), and APC/MCAK (F) -depleted egg extracts or egg extracts supplemented with purified recombinant N-APC (G) or EB1C (H). (I) Quantification of structures formed from sperm nuclei in cycled egg extracts as indicated scored 80 min after exit from interphase. At least 50 mitotic structures were scored for each extract. Data are presented from one representative experiment. (J and K) Purification of recombinant *Xenopus* N-APC ($APC^{1–1,450}$; J) and GST-EB1C ($EB1^{165–268}$; K). Initial *E. coli* lysates encoding N-APC or EB1C (lane 1) and GST–N-APC or GST-EB1C after affinity purification over immobilized glutathione (lane 2). (L) Immunoprecipitates with an APC antibody from CSF-arrested egg extracts containing sperm nuclei (lanes 3 and 4) or supplemented with N-APC (lanes 1 and 2) or EB1C (lanes 5 and 6) with affinity-purified rabbit anti-APC IgG and probed with APC, BubR1, EB1, or GST (for GST–N-APC and GST-EB1C) antibodies. FL, extracts after immunoprecipitation; IP, immunoprecipitates. A threefold higher proportion of the bead-bound fraction relative to the depleted extracts was analyzed. Bars, 10 μm.

formed in the presence of GST–N-APC displayed severe chromosome alignment defects (Fig. 5, G and I), which is a phenotype similar to APC depletion (Fig. 3, B and I). This result provides further evidence that the chromosome alignment defect observed upon APC depletion is specific. Immunoprecipitation analysis revealed that the N-APC fragment indeed oligomerized with endogenous APC proteins (Fig. 5 L, lane 1) as expected (Joslyn et al., 1993; Su et al., 1993). However, APC and EB1 interaction was decreased upon the addition of N-APC to *Xenopus* egg extracts (Fig. 5 L; compare EB1 in lanes 1 and 3), as has been shown in mammalian cells (Green et al., 2005), although purified APC without the N terminus can efficiently bind to EB1 in vitro (Fig. 6 A). Furthermore, the addition of N-APC in egg extracts almost completely eliminated BubR1 interaction with endogenous APC (Fig. 5 L; compare BubR1 in lanes 1 and 3).

Our results suggest that APC/EB1 interaction is essential for BubR1–APC/EB1 complex formation at attached kinetochores. One possibility is that EB1 may enhance the interaction of microtubules and APC at plus ends of microtubules. To test this possibility, a purified recombinant C-terminal EB1 fragment EB1C (Fig. 5 K), which cannot bind to microtubules, was added to *Xenopus* egg extracts. The EB1C still contains a conserved domain that is known to interact with APC (Berrueta et al., 1998). EB1C was indeed coimmunoprecipitated with APC (Fig. 5 L, EB1C in lane 5). However, this fragment substantially decreased APC association with endogenous EB1 (Fig. 5 L, compare EB1 in lanes 3 and 5) and BubR1 (Fig. 5 L, compare BubR1 in

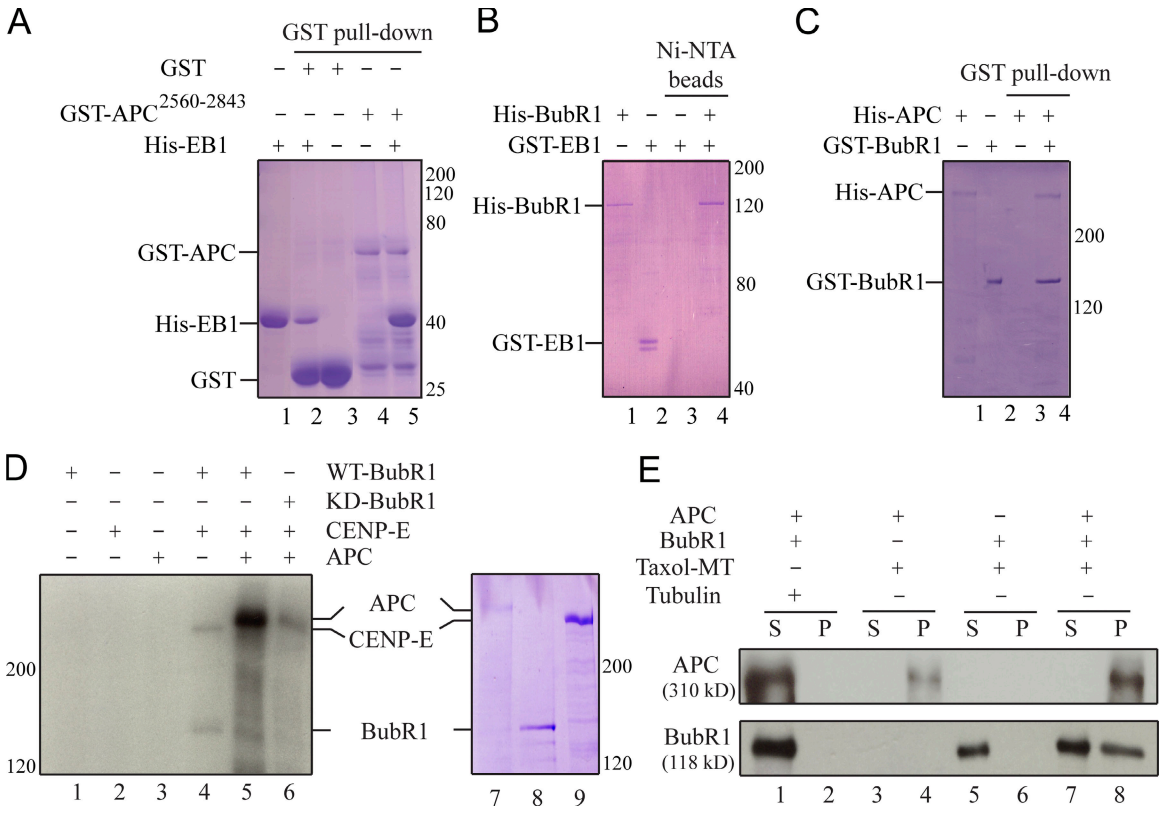


Figure 6. **BubR1 phosphorylates APC and forms a ternary complex with APC and microtubules in vitro.** (A–C) In vitro binding assay using purified proteins as indicated. Purified recombinant proteins were incubated with glutathione–Sepharose 4B (A and C) or Ni-NTA agarose (B) beads as indicated. Protein pulldown with the beads was assayed by SDS-PAGE and Coomassie blue staining. (D) APC phosphorylation by BubR1. In vitro kinase assay was performed with a combination of purified recombinant APC (lane 7), BubR1 (lane 8), and CENP-E (lane 9) as indicated. (E) BubR1, APC, and microtubules form a ternary complex. After centrifugation through a 40% sucrose cushion, BubR1–APC–taxol microtubule complex formation was assayed by immunoblotting. S, supernatant; P, pellet.

lanes 3 and 5). Furthermore, metaphase chromosome alignment was disrupted upon the addition of EB1C (Fig. 5, H and I). These results suggest that the complex formation of BubR1–APC/EB1 is important for its function in chromosome alignment.

### BubR1 interacts and phosphorylates APC in vitro

We have shown that BubR1 can form a complex with APC and EB1 in *Xenopus* egg extracts (Fig. 2). To determine whether the BubR1–APC/EB1 association is direct, full-length recombinant BubR1, APC, and EB1 proteins were purified from *E. coli* and/or insect Sf9 cells (using the Baculovirus system). Recombinant C-terminal APC$^{2,560–2,843}$ (with the known EB1-binding domain; Su et al., 1995) effectively pulled down full-length recombinant protein EB1 (Fig. 6 A; compare lane 2 with lane 5), whereas BubR1 protein did not (Fig. 6 B, lane 3), demonstrating that BubR1 cannot directly bind to EB1. To test whether BubR1 can directly bind to APC, purified GST-BubR1 was incubated with His-APC. As shown in Fig. 6 C, a considerable amount of APC was precipitated with GST-BubR1 but not GST alone (compare lane 3 with lane 4). This demonstrates that BubR1 can directly bind to APC.

We next determined whether APC is a substrate for BubR1. An in vitro kinase assay was performed using purified APC, BubR1, and CENP-E (Fig. 6 D, lanes 7–9), the activator of BubR1 kinase (Mao et al., 2003, 2005). This revealed that BubR1 phosphorylated APC efficiently (Fig. 6 D, lane 5). Furthermore, APC was a much better substrate for BubR1 compared with CENP-E and BubR1 itself (Fig. 6 D, compare lane 4 with lane 5). This was not the result of a contaminating kinase because in a parallel assay, BubR1 containing a K787R point mutation in the ATP-binding pocket was much less active in APC phosphorylation (Fig. 6 D, lane 6). This is consistent with a previous study that showed that BubR1/Bub1 can phosphorylate an APC fragment in vitro (Kaplan et al., 2001). We conclude that BubR1 can phosphorylate APC efficiently and with very high specificity in vitro.

### BubR1 forms a ternary complex with APC and microtubules in vitro

To determine whether microtubule-bound APC can still interact with BubR1, pure BubR1 and APC proteins were incubated with unpolymerized tubulin or taxol microtubules. The microtubules and proteins bound to them were recovered by sedimentation through a sucrose cushion. BubR1 did not pellet on its own with taxol-stabilized microtubules (Fig. 6 E, lane 6). In contrast, after the addition of full-length APC, a protein known to bind to microtubules (Deka et al., 1998; Zumbrunn et al., 2001), all APC and a substantial amount of BubR1 cosedimented with microtubules (Fig. 6 E, lane 8). Thus, the interaction between BubR1 and microtubule-bound APC produced a ternary complex, and this is essential for stable kinetochore microtubule attachment.

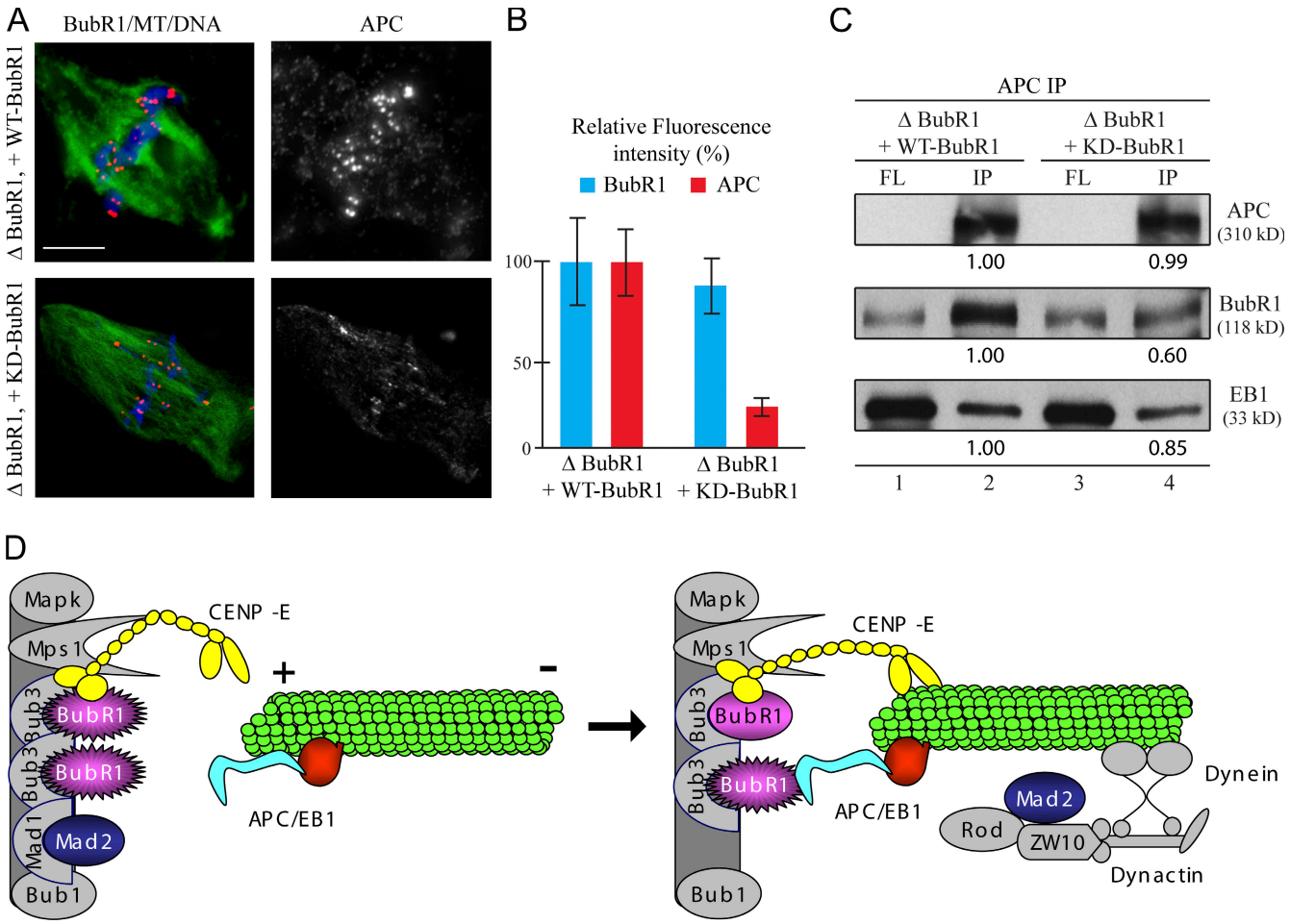


Figure 7. **BubR1 kinase activity is essential for the efficient recruitment of APC onto kinetochores in *Xenopus* egg extracts.** (A) BubR1-depleted egg extracts containing sperm nuclei were cycled through interphase and arrested with CSF activity at the following metaphase. Recombinant WT-BubR1 (top) or KD-BubR1 (bottom) was added. Kinetochore recruitment of BubR1 (left; red), APC (right), and microtubules (left; green) was visualized by immunofluorescence with specific antibodies. Chromatin was visualized with DAPI (left; blue). (B) Quantification of the relative BubR1 and APC intensity at kinetochores in A. Error bars represent SEM. (C) Immunoprecipitates with an APC antibody from BubR1-depleted CSF egg extracts containing sperm nuclei and supplemented with purified WT-BubR1 (lanes 1 and 2) or KD-BubR1 (lanes 3 and 4) and probed with APC (top), BubR1 (middle), and EB1 (bottom) antibodies. FL, extracts after immunoprecipitation; IP, immunoprecipitates. A threefold higher proportion of the bead-bound fraction relative to the depleted extracts was analyzed. (D) Model: BubR1 is recruited onto unattached kinetochores. Microtubule-associated proteins APC/EB1 bind to the plus ends of microtubules. After the initial capture of microtubules by kinetochores, the interaction between BubR1 and APC/EB1 stabilizes kinetochore microtubule attachment, in which BubR1 might directly phosphorylate APC. Bar, 10 μm.

### BubR1 kinase activity is essential for the efficient recruitment of APC onto kinetochores in *Xenopus* egg extracts

We have concluded that complex formation between BubR1 and APC/EB1 is necessary for their functions in kinetochore microtubule attachment and metaphase chromosome alignment. To further confirm whether APC–BubR1 interaction is essential for metaphase chromosome alignment, we examined the amount of APC at kinetochores in BubR1-depleted egg extracts supplemented with either WT or KD-BubR1 because only WT- but not KD-BubR1 can restore the chromosome alignment defect in BubR1-depleted egg extracts (Fig. 1, H–J). Immunofluorescence analysis has revealed that the amount of APC but not BubR1 at kinetochores was considerably reduced in BubR1-depleted egg extracts supplemented with KD-BubR1 (Fig. 7, A and B). Furthermore, APC was immunoprecipitated from egg extracts depleted of endogenous BubR1 and supplemented with a similar amount of purified WT- or KD-BubR1 recombinant proteins. Immunoprecipitates obtained using the APC antibody contained comparable levels of APC and EB1 under both conditions (Fig. 7 C, compare APC with EB1 in lanes 2 and 4). However, ~40% less KD-BubR1 was coimmunoprecipitated with APC compared with WT-BubR1 (Fig. 7 C, compare BubR1 in lanes 2 and 4), indicating that APC phosphorylation by BubR1 might be necessary for APC function in metaphase chromosome alignment in *Xenopus* egg extracts.

## Discussion

The mitotic checkpoint functions for BubR1 kinase are well established: one is an evolutionary conserved Mad3-like role as part of a diffusible inhibitor (Sudakin et al., 2001; Tang et al., 2001; Mao et al., 2003); added to this is a CENP-E–dependent kinase activity that is essential for one or more local roles as

a kinetochore-bound catalytic facilitator (Mao et al., 2003, 2005; Weaver et al., 2003). Using antibody addition, immunodepletion, and reconstitution with purified recombinant proteins in *Xenopus* egg extracts, which are naturally arrested in metaphase, we have demonstrated that BubR1 is necessary for metaphase chromosome alignment, which is consistent with recent studies regarding the involvement of BubR1 in kinetochore microtubule capture and attachment in mammalian cultured cells (Johnson et al., 2004; Lampson and Kapoor, 2005). Furthermore, we show that BubR1 kinase activity is essential for its role in kinetochore microtubule attachment.

The alignment defects we observed do not appear to result from a general kinetochore assembly defect: (1) the addition of inhibitory antibody or reconstitution with KD-BubR1 disrupts chromosome alignment (this study) without removing kinetochore-associated motor CENP-E (Mao et al., 2003) as the immunodepletion of BubR1 does; (2) using an immunodepletion and reconstitution approach in *Xenopus* egg extracts, it has been shown that the depletion of BubR1 has no effect on the kinetochore localization of Mps1 and CENP-A, and the addition of either WT- or KD-BubR1 to BubR1-depleted egg extracts restores BubR1, Plx1, and 3F3/2 signals at kinetochores (Wong and Fang, 2006); and (3) immunodepletion of BubR1, APC, or EB1 from *Xenopus* egg extracts does not affect kinetochore localization of the Ndc80 complex (unpublished data). Thus, based on a combination of the in vitro results presented in this study and prior in vivo BubR1 RNAi phenotypic analysis (Lampson and Kapoor, 2005), we propose that BubR1 and its kinase activity are directly involved in regulating stable kinetochore microtubule attachment and metaphase chromosome alignment. This also resolves a difference in the involvement of CENP-E in metaphase chromosome alignment between the *Xenopus* and mammalian systems. Using immunodepletion from and antibody addition to *Xenopus* egg extracts, the kinetochore-associated kinesin-like motor protein CENP-E has been shown to be essential for positioning chromosomes at the metaphase plate (Wood et al., 1997). In contrast, most mammalian cells without CENP-E have a robust metaphase plate with only a few chromosomes abnormally close to the spindle poles (McEwen et al., 2001; Martin-Lluesma et al., 2002; Putkey et al., 2002). The key underlying difference between two systems could simply be the activation of BubR1 kinase by CENP-E. The mammalian BubR1 has a basal level kinase activity in the absence of CENP-E (Weaver et al., 2003), whereas its *Xenopus* homologue does not have any kinase activity without its activator CENP-E (Mao et al., 2003). Indeed, in the presence of a motorless CENP-E fragment that can constitutively activate BubR1, the majority of chromosomes congress to the metaphase plate in the *Xenopus* egg extracts depleted of endogenous CENP-E (Mao et al., 2005).

The plus end–tracking protein EB1 and its binding partner APC have previously been implicated in chromosome behavior in mammalian cultured cells (Kaplan et al., 2001; Green and Kaplan, 2003; Tighe et al., 2004; Green et al., 2005). In the present study, we show that APC and EB1 cooperate with the mitotic checkpoint kinase BubR1 to maintain stable kinetochore microtubule attachment and metaphase chromosome alignment (Fig. 7 D). BubR1 is recruited onto unattached kinetochores (Chan et al., 1999; Chen, 2002; Mao et al., 2003). The microtubule-associated proteins APC and EB1 bind to plus ends of microtubules (Rogers et al., 2002). After the initial capture of microtubules by kinetochores (through an as yet unknown mechanism), the interaction between BubR1 and APC/EB1 stabilizes kinetochore microtubule attachment, in which BubR1 might directly phosphorylate APC. Evidence for this model includes the following: (1) BubR1 forms a complex with APC and EB1 in *Xenopus* egg extracts; (2) the codepletion of BubR1, APC, and EB1 has no additive effect on mitotic spindle defects; (3) replacing endogenous BubR1 with KD-BubR1 in egg extracts considerably decreases the amount of APC at kinetochores and produces a chromosome alignment defect; (4) N-APC and EB1C fragments, which disrupt the complex formation of BubR1–APC/EB1, produce a chromosome alignment defect in *Xenopus* egg extracts; (5) BubR1 forms a ternary complex with APC and microtubules in vitro; and (6) compared with another BubR1-binding partner, CENP-E, APC is a much better substrate for BubR1, indicating that APC might be one of the physiological substrates of BubR1. Finally, BubR1 localization to kinetochores in APC/EB1-depleted egg extracts was not impaired, suggesting that chromosome alignment defects in the absence of BubR1 or APC/EB1 reflect the formation of a functional complex among these proteins.

APC has been shown to interact with mitotic checkpoint proteins Bub1/Bub3 (Kaplan et al., 2001) and BubR1 (this study). Whether APC has a role in mitotic checkpoint signaling is controversial. The depletion of APC has been reported to cause chromosome misalignment in metaphase and missegregation in anaphase (Green et al., 2005), indicating a defect in the mitotic checkpoint response. In addition, a recent study has shown that cells transfected with APC-targeting siRNA accumulated 1.84-fold and 1.66-fold less Bub1 and BubR1, respectively (Dikovskaya et al., 2007). However, Draviam et al. (2006) have shown high levels of kinetochore-bound Mad1, Mad2, Mps1, Bub1, and BubR1 checkpoint proteins in control-, EB1-, or APC-depleted prometaphase cells, which is consistent with an intact mitotic checkpoint. They suggest that instead of a checkpoint defect, chromosome missegregation in APC- and EB1-depleted cells is caused by misorientation and reduced stretching of aligned sister kinetochores, errors that are detected poorly, if at all, by the mitotic checkpoint (Draviam et al., 2006). We have shown that the mitotic checkpoint is activated in APC- and EB1-depleted *Xenopus* egg extracts with high levels of BubR1 kinase activity and kinetochore-bound BubR1 and Mad2 (Fig. 4). Furthermore, APC- and EB1-depleted egg extracts have a chronically activated mitotic checkpoint response even in the presence of spindle microtubules (in the absence of nocodazole), supporting our conclusion that there is improper kinetochore microtubule attachment in the absence of APC and EB1. However, our results do not exclude the possibility that APC might have a nonessential enhancement role in mitotic checkpoint signaling.

Our current studies confirm by completely independent means and extend the evidence that both BubR1 and APC/EB1 individually play a role in metaphase chromosome alignment. In addition, our egg extracts and in vitro results have important

implications that complex formation between BubR1 and APC/EB1 is essential for the successful formation of stable kinetochore microtubule attachment, providing a potential link in stable kinetochore microtubule attachment. Consistent with this idea, BubR1$^{+/-}$ APC$^{min/+}$ compound mice have been shown to develop 10 times more colonic tumors than APC$^{min/+}$ mice (Rao et al., 2005), demonstrating that there is indeed a functional interaction between BubR1 and APC in vivo. Furthermore, APC has been shown to associate with MCAK at the centromere/kinetochore region in *Xenopus* egg extracts (Banks and Heald, 2004). MCAK is thought to be involved in the depolymerization of improperly attached microtubules (merotelic and syntelic microtubule attachment at kinetochores) to ensure accurate chromosome segregation (Kline-Smith et al., 2004). MCAK is one of the known substrates of Aurora B (Andrews et al., 2004; Lan et al., 2004), and its microtubule-depolymerizing activity is reduced upon Aurora B phosphorylation in vitro (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). The kinetochore-microtubule destabilization phenotype upon BubR1 and APC depletion can be partially suppressed by Aurora kinase inhibition in both mammalian cultured cells (Lampson and Kapoor, 2005) and *Xenopus* egg extracts (Fig. 5). These results indicate that BubR1, APC/EB1, Aurora B, and MCAK might cooperate to ensure proper kinetochore microtubule attachment.

## Materials and methods

### *Xenopus* APC and EB1 purification, antibody production, and immunoblotting

The full-length cDNA clone for *Xenopus* APC was a gift from B. Gumbiner (Memorial Sloan-Kettering Cancer Center, New York, NY), and the full-length *Xenopus* EB1 cDNA clone was provided by Y. Tsuchiya (Toho University School of Medicine, Tokyo, Japan). *Xenopus* APC and EB1 have 75.8% and 83.3% identities to human homologues, respectively.

APC fragment (APC$^{2,073–2,472}$) and full-length EB1 proteins were expressed in *E. coli* with a His tag at the N terminus and were purified over Ni–nitrilotriacetic acid (NTA) columns. Purified proteins were used to raise antibodies in rabbits (for APC) and chickens (for EB1), and sera were affinity purified over immobilized APC and EB1 fusion proteins.

Immunoblots were blocked with TBST (20 mM Tris−HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween) containing nonfat dry milk and probed with affinity-purified primary antibodies in TBST. Primary antibodies were visualized using an HRP-labeled goat anti–rabbit IgG or a goat anti–chicken IgY secondary antibody and ECL. Image software (Scion) was used to quantify the immunoreactivity.

### *Xenopus* egg extracts

CSF-arrested extracts were prepared from unfertilized *Xenopus* eggs as previously described (Murray, 1991). Checkpoint extracts were prepared from these by incubation with ∼9,000 demembranated sperm nuclei/μl and 10 μg/ml nocodazole (Minshull et al., 1994). Exit from CSF arrest was induced by the addition of 0.4 mM $CaCl_2$.

For immunodepletion, 100 μg of affinity-purified BubR1, APC, EB1, Aurora B (a gift from H. Funabiki, The Rockefeller University, New York, NY), and MCAK (provided by C. Walczak, Indiana University, Bloomington, IN) antibodies or nonimmune rabbit IgG or chicken IgY was bound to 100 μl Dynal bead protein A (in the case of chicken antibody, the protein A beads were precoupled to 50 μg AffiniPure rabbit anti–chicken IgY antibodies [Jackson ImmunoResearch Laboratories]). 100 μl CSF egg extracts were added for 1 h at 4°C. For antibody addition experiments, affinity-purified antibodies or nonimmune chicken IgY were added to CSF egg extracts at 100 μg/ml.

Demembranated sperm was added to a portion of each extract, rhodamine-labeled bovine brain tubulin was added at 1/300 μl of extract, and exit from metaphase arrest was induced by the addition of $Ca^{2+}$. Cell cycle progress of egg extracts was followed by fluorescence microscopic examination of 1-μl aliquots squashed under a coverslip. 80 min after exiting from metaphase, 0.5 vol of the appropriate egg extracts was added and incubated for an additional 60–120 min. M-phase structures accumulating in egg extracts were scored in squashed samples. Bipolar spindles with all chromosomes aligned at the spindle equator were scored as bipolar aligned, whereas bipolar spindles with scattered chromosomes were counted as bipolar misaligned. Monopolar and other mitotic structures were scored as other. At least three independent depletion experiments were performed.

Immunofluorescence with *Xenopus* egg extracts was performed as previously described (Wood et al., 1997). For immunofluorescence, all images in each experiment were collected on the same day using identical exposure times. Image acquisition and data analysis were performed at room temperature using an inverted microscope (IX81; Olympus) with a 60× NA 1.42 plan Apo oil immersion objective lens (Olympus), a monochrome CCD camera (Sensicam QE; Cooke), and the Slidebook software package (Olympus). The APC kinetochore fluorescence intensity was quantified using Image software (Scion).

### Immunoprecipitation

Affinity-purified antibodies were coupled to Dynal beads, and the beads were washed twice with lysis buffer. The beads were then incubated at 4°C for 1 h with egg extracts that had been incubated at room temperature for 30 min with or without sperm nuclei and nocodazole. After mixing, the beads were washed twice with PBS buffer and twice with PBS plus 0.5 M NaCl. The immunoprecipitates were then solubilized in SDS sample buffer and subjected to immunoblot analysis.

### Sucrose density gradient sedimentation

200 μl of high speed supernatant of CSF-arrested egg extracts was loaded onto a 10-ml 5–15% continuous sucrose gradient and spun for 16 h at 32,000 rpm in a SW41Ti rotor (Beckman Coulter) at 4°C. 500 μl of fractions were collected from each gradient.

### Recombinant protein production

*Xenopus* APC was expressed in insect cells infected with a baculovirus encoding APC and purified over immobilized Ni-NTA agarose. *Xenopus* GST–N-APC and *Xenopus* His-EB1 and -EB1C were expressed in *E. coli* and purified over glutathione–Sepharose beads or Ni-NTA agarose beads, respectively.

### In vitro kinase assay

For in vitro kinase assays, recombinant BubR1 and CENP-E were incubated with or without purified APC at room temperature for 30 min with 25 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 200 μM ATP, and 1 μCi γ-[$^{32}$P]ATP and were assayed for kinase activity.

### BubR1/APC microtubule cosedimentation assay

10 nM of purified BubR1 and/or APC was mixed with 1 μM taxol-stabilized microtubules or unpolymerized tubulin in BRB80 and incubated in room temperature for 30 min. Binding reactions were centrifuged over 40% sucrose/BRB80 at 10,000 rpm for 30 min at 35°C. Recombinant proteins in the pellet and supernatant were analyzed by immunoblotting.

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