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Microtubule capture by CENP-E silences mitotic checkpoint signaling

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Running title: Silencing the mitotic checkpoint

Summary

The mitotic checkpoint is the major cell cycle control mechanism for maintaining diploid chromosome content. Prevention of premature onset of anaphase requires activation at unattached kinetochores of the BubR1 kinase, which acts with other components to generate a diffusible stop anaphase inhibitor. Not only does direct binding of BubR1 to the centromere-associated kinesin family member CENP-E activate its essential kinase, binding of a motorless fragment of CENP-E is shown here to constitutively activate BubR1 bound at kinetochores, producing checkpoint signaling that cannot be silenced by spindle microtubule capture. Using purified BubR1, microtubules and CENP-E, microtubule capture by the CENP-E motor domain is shown to silence BubR1 kinase activity in a ternary complex of BubR1-CENP-E-microtubule. Together, this reveals that CENP-E is the signal transducing linker responsible for silencing mitotic checkpoint signaling through its capture at kinetochores of spindle microtubules.

In order to assure accurate sister chromatid segregation, eukaryotic organisms have evolved a "mitotic checkpoint" to prevent premature advance to anaphase prior to successful attachment of every chromosome to microtubules of the mitotic spindle. Chromosome instability (CIN) leading to an abnormal chromosome number (aneuploidy) is consistently associated with the loss of the function of the mitotic checkpoint in human cancers (*1*). Genetics in yeast initially identified seven components of the mitotic checkpoint, Mad1-3 (*2*), Bub1-3 (*3*), and Mps1 (*4*). There are vertebrate homologs of all of these except Bub2 (*5-10*). Additional required contributors without yeast counterparts are known in metazoans. Without the kinetochore-associated microtubule motor protein

CENP-E, the checkpoint cannot be established or maintained *in vitro* (*11*) or in mice (*12*). Inhibition of ZW10 and Rod by mutation (*13*) or antibody injection (*14*) have revealed both also to be required for checkpoint signaling. Initially demonstrated for Mad2 (*5*), all have now been identified to be recruited onto unattached kinetochores (*15*) where they generate a diffusible signal to prevent anaphase onset. Prior to spindle attachment, a Mad1/Mad2 complex is stably targeted to kinetochores where it recruits additional Mad2 that is converted into a rapidly released, inhibitory form (*16*) (including the possible assembly of a complex with other checkpoint proteins (*17, 18*)). The activated inhibitor(s) binds to and sequesters Cdc20, a specificity factor required for an E3 ubiquitin ligase called the anaphase-promoting complex/Cyclosome (APC/C) to recognize substrates (including the securins) whose destruction is required for advance to anaphase (*15*).

Although the basic plan of the signaling cascade is established, how spindle microtubule binding to kinetochores silences mitotic checkpoint signaling is not established. BubR1, the vertebrate homolog of yeast Mad3, has acquired a kinase domain in every species (including Drosophila, Xenopus, mice, and human) that also has a CENP-E homolog. BubR1 kinase activity is essential for the metazoan mitotic checkpoint and is directly stimulated by CENP-E binding to it (*19*). Since CENP-E is also one of the components directly responsible for capture and stabilization of spindle microtubules by kinetochores (*12, 20*), it is a candidate for the signal transducing linker responsible for silencing checkpoint signaling at each kinetochore through capture by its motor domain of spindle microtubules.

If CENP-E capture of spindle microtubules is required for mitotic checkpoint signal silencing, replacement of CENP-E with a motorless variant sufficient for activating the BubR1 kinase should produce a chronic checkpoint signal independent of kinetochore capture of spindle microtubules. After immunodepletion of endogenous CENP-E from Xenopus egg extracts, addition of such a motorless CENP-E (the carboxyl terminal 700 amino acid fragment of the 2954 amino acid CENP-E (CENP-E_{tail}, Figure 1A and Supplemental Figure 1A)) lead to recruitment of both it and BubR1 to kinetochores (Figure 1B). Bipolar spindles with aligned chromosomes formed at high efficiency in undepleted or mock depleted extracts (Supplemental Figure 1B, left panels; Supplemental Figure 1C). After removal of CENP-E (*21, 22*) or replacement of endogenous CENP-E with CENP-E_{tail} most chromosomes still attached to spindle microtubules, although a proportion were misaligned chromosomes (Supplemental Figure 1B, right panels; Supplemental Figure 1C), consistent with a contribution of the CENP-E motor domain in achieving or maintaining metaphase chromosome alignment.

BubR1 kinase activity is CENP-E dependent (*19*). In the presence of endogenous full length CENP-E, both BubR1 kinase activity (Figure 1D, lanes 1 and 2) and mitotic checkpoint signaling (Figure 1C) were activated in egg extracts only when kinetochore attachment was blocked by inhibition of microtubule assembly. However, after depletion of BubR1 and CENP-E and restoration of BubR1 with GST-tagged BubR1, addition of CENP-E_{tail} activated BubR1 kinase (measured with affinity recovery of BubR1 with GST antibody beads) to the same levels as in extracts containing endogenous CENP-E (Figure 1D, lane 1 vs 4). BubR1 kinase activity was silenced by kinetochore microtubule attachment in extracts with full length CENP-E (Figure 1D, lane 2). However, CENP-E_{tail}

stimulated BubR1 kinase activity was undiminished after spindle assembly (Figure 1D, lane 5), producing chronic mitotic checkpoint signaling whether or not spindles were assembled, as revealed by continued Cdc2 kinase activity and condensed chromosomes (Figure 1C, bottom two rows).

Mad2, an essential mitotic checkpoint component, binds preferentially to unattached kinetochores that are producing the stop anaphase inhibitor (*5, 24*). In extracts in which endogenous CENP-E was replaced with full length recombinant CENP-E, Mad2 was released from kinetochores upon microtubule attachment, while BubR1 association remained (Supplemental Figure 2, left panel). After replacement of CENP-E with the microtubule capture deficient CENP-E_{tail}, Mad2 association was undiminished at the unreplicated kinetochores assembled on the chromosomes from a haploid sperm nucleus despite attachment and alignment of most chromosomes (Supplemental Figure 2, center panel) and on duplicated, unattached kinetochores produced after cycling through interphase (Figure 2A). Mad2 remained not only on the unattached sister kinetochores (Figure 2A), but also those that were bi-polar attached (Figure 2A). Alignment along the spindle axis and increased sister kinetochore separation (relative the spacing after nocodazole addition) indicated that some of these kinetochores were under tension developed following successful CENP-E independent, bi-polar attachment (Figure 2A, insets, 2B). Nevertheless, Mad2 remained associated with these attached kinetochores at levels very similar to that of unattached kinetochores (Figure 2A, 2B). Therefore, while other components can mediate spindle microtubule capture at kinetochores and generate tension between sister kinetochores in the absence of CENP-E, silencing of BubR1 kinase activity and Mad2 recruitment at kinetochores requires an activity of CENP-E

beyond its kinetochore binding domain. This was also the case in human cells. Human CENP-E_{tail} (Flag:hCENP-E_{tail}, aa 1572-2664) accumulated at kinetochores (Figure 2C, left panel) in transfected HeLa cells. Further, in such cells, Mad2 remained not only on unattached kinetochores (Figure 2C, center panel), but also those that were bi-polar attached (Figure 2C, center panel, inset, judging by increased sister kinetochore separation), but not at kinetochores of aligned chromosomes in the CENP-E containing nontransfected cells (Figure 2C, right panel).

To directly determine whether microtubule capture by CENP-E silences the BubR1 kinase-dependent mitotic checkpoint signaling, microtubules resistant to disassembly were assembled from pure tubulin in the presence of GMPCPP, a slowly hydrolysable GTP analogue (*25*). As expected (*19*), purified BubR1 autophosphorylated itself, but only in the presence of CENP-E (Figure 3A, compare lanes 1 and 3). CENP-E-activated BubR1 also phosphorylated CENP-E (Figure 3A, lane 3). Subsequent addition of GMPCPP stabilized microtubules strongly inhibited this CENP-E-dependent BubR1 kinase activity in a dose dependent manner (Figure 3A, compare lanes 3 and 4). Kinase inhibition was specific for addition of assembled microtubules, since neither GMPCPP alone nor high concentrations of tubulin affected BubR1 kinase activity (Figure 3A, lanes 9 and 10). Further, addition of GMPCPP stabilized microtubules had no effect on BubR1 kinase activity stimulated by CENP-E_{tail} (Figure 3B, compare lanes 3 and 4). BubR1 had no microtubule affinity on its own. None co-pelleted with microtubules (Figure 4A, lane 3) and none was found microtubule associated after fixation and visualization using fluorescently tagged BubR1 antibodies (Figure 4B). In contrast, in low concentrations of ATP in which the CENP-E motor domain is known to be largely microtubule bound (*26*),

after addition of full-length CENP-E, but not CENP-E_{tail}, a significant proportion of BubR1 co-sedimented with microtubules (Figure 4A, lane 2). CENP-E bound in a dot-like pattern along those microtubules, with BubR1 also targeted only to these CENP-E foci (Figure 4B).

Thus, with purified components, microtubule capture by CENP-E produced a ternary complex of the captured microtubule, CENP-E and BubR1 and this silenced the BubR1 kinase activity known to be essential for checkpoint signaling. Added to this, using Xenopus egg extracts and human cultured cells, motorless CENP-E_{tail} containing both the BubR1 and kinetochore targeting domains was shown to chronically activate BubR1 kinase in a way that could not be silenced by microtubule capture by components other than CENP-E, even for kinetochore pairs that aligned and developed tension between bi-oriented sister kinetochores. This also offers an explanation for a perplexing finding in flies, which have two CENP-E-like genes: loss of function mutation in one leads to a chronically activated checkpoint, while mutation in the other leads to a checkpoint deficiency and premature anaphase (*27*). Thus, in flies the bi-functional role of CENP-E in the mitotic checkpoint (activating and silencing) is apparently achieved by two CENP-E products each providing one of the two functions.

Despite a previous controversy as to whether the mitotic checkpoint is silenced by microtubule attachment (*28*) or the subsequent tension generated (*29*) and whether activities of subsets of the known components are selectively silenced by one or the other (*30, 31*), it is now clear that CENP-E is the signal transducing linker responsible for silencing of BubR1-dependent mitotic checkpoint signaling through its capture of spindle

microtubules and the corresponding inhibition of BubR1 kinase irrespective of microtubule capture and tension developed by other kinetochore components.

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Materials and Methods

Fusion Protein Expression and Immunoblotting

XCENP-E tail (aa 2397-2954) were produced as 6His fusion protein using the pRSET expression plasmid. Following induction with IPTG, recombinant protein was purified over Ni-NTA agarose (Qiagen).

Immunoblot were blocked with TBST (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween) containing non fat dry milk and then probed with affinity-purified primary antibodies in TBST. Primary antibodies were visualized using a horseradish peroxidase-labeled secondary antibody and ECL (Amersham).

Xenopus Egg Extracts and Spindle Assembly in vitro

CSF-arrested extracts were prepared from unfertilized Xenopus eggs as previously described (*32*). Rhodamine-labeled bovine brain tubulin was added at 1 µl/300 µl of extracts. Immunodepletion of BubR1 or CENP-E, affinity purified anti-BubR1 or anti-CENP-E antibodies were used and carried as previously described (*19*). Demembranated sperm were added to a portion of each extract, and exit from metaphase arrest induced by addition of $CaCl_2$. Progression of extracts through the cell cycle was monitored by fluorescence microscopic examination of 1 µl aliquots squashed under a coverslip (*32*). At 80 min following exit from metaphase, one half volume of the appropriate extracts were added and the reaction incubated for an additional 60-120 min. M-phase structures accumulating in extracts were scored in squashed samples taken after 160 min total elapsed time. Immunofluorescence with Xenopus egg extracts was performed as previous described (*19*).

In vitro Kinase Assay

For kinase assay, GST-BubR1 immunoprecipitates were incubated 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 100 μg histone H1 as an exogenous substrates. Recombinant BubR1 were incubated with or without CENP-E, GMPPNP microtubules and then assayed autokinase activity.

Cell Culture, Transfection, and Immunfluorescence

Hela H71-9 (Hela cells stably expressing Mad2-ECFP) were grown in DMEM supplemented with 10% Fetal Bovine Serum. Transfections were done using Effectene based on manufacture protocol (Qiagen).

For immunofluorescence, cells grown on poly-L-lysine-coated coverslips were washed once with MTSB (microtubule stabilizing buffer, 100 mM Pipes, 1 mM EGTA, 1 mM $MgSO_4$, and 30% of glycerol), extracted with 0.5% Triton-X100 in MTSB for 5 minutes, fixed with 4% formaldehyde in MTSB for 10 minutes, and blocked in TBS containing 0.5% Tween-20 and 1% BSA (Sigma) for 1 hour. Coverslips were exposed to primary antibodies diluted in blocking buffer for 1 hour, and to secondary antibodies (Jackson ImmunoResearch).

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Figure Legends

Figure 1. CENP-E_{tail} domain is sufficient for activating BubR1 kinase activity and the mitotic checkpoint signaling.

(A) Xenopus CENP-E protein structure showing the relative position of the N-terminal microtubule motor domain, the rod domain, and the C-terminal kinetochore binding tail domain.

(B) Recombinant CENP-E_{tail} can be recruited onto kinetochores. CSF-arrested egg extracts were mock depleted (left panel), or CENP-E depleted and supplemented with CENP-E_{tail} (right panel). 30 minutes after addition of sperm nuclei, extracts were observed by immunofluorescence with anti-CENP-E and anti-BubR1 antibodies, and chromatin was visualized with DAPI.

(C) CENP-E_{tail} is sufficient for activating the mitotic checkpoint signaling. Egg extracts were mock depleted (rows 1 and 2), or CENP-E depleted (row 3), and then supplemented with recombinant CENP-E_{tail} (rows 4 and 5). After incubation with sperm nuclei and with or without nocodazole as indicated, calcium was added to the extracts to inactivated CSF activity. Aliquots were taken from each extract at indicated time and then assayed by autoradiography for Cdc2 kinase activity using added histone H1 as a substrate (left) and chromatin condensation (right).

(D) CENP-E_{tail} itself is sufficient for BubR1 kinase activation in Xenopus egg extracts. BubR1 was immunodepleted from CSF-arrested egg extracts and GST-BubR1 was added to the level of endogenous BubR1. Sperm nuclei and nocodazole were added to extracts with either CENP-E (mock depleted) or CENP-E_{tail} (CENP-E depleted and supplemented with CENP-E tail) as indicated. After 30 min, GST-BubR1 was immunoprecipitated

using GST antibody and immunoblotted with BubR1 antibody (bottom) or kinase activity measured after addition of histone H1 and [γ-^{32}P]-ATP (top).

Figure 2. Microtubule capture by CENP-E is required for preventing Mad2 kinetochore recruitment.

(A) CENP-E-depleted extracts containing sperm nuclei were cycled through interphase and arrested at following metaphase. CENP-E_{tail} recombinant proteins were added. 60 min after addition of nocodazole or not as indicated, the kinetochore recruitment of Mad2 and spindle microtubules were visualized by specific antibodies, and chromatin was visualized with DAPI.

(B) Quantification of the spacing between sister kinetochores and the normalized integrated intensity of Mad2 signals at kinetochores from (B).

(C) Flag:hCENP-E_{tail} and Mad2:CFP were observed by immunofluorescence with anti-flag and anti-GFP antibodies respectively, and chromatin was visualized with DAPI in Mad2:CFP HeLa line overexpressing with Flag:hCENP-E_{tail}.

Figure 3. Addition of microtubules to purified CENP-E and BubR1 inactivates CENP-E-dependent stimulation of BubR1 kinase activity.

(A) In vitro kinase assay were performed with combination of purified GST-BubR1 (lane a), CENP-E (lane b), and in addition of GMPCPP microtubules range from 1 μM to 0.1 nM (lanes 4 to 8), tubulin (1 μM, lane 9), or GMPCPP (33 μM, lane 10).

(B) In vitro kinase assay were performed with combination of purified CENP-E_{tail} and GST-BubR1 in the presence or absence of GMPCPP microtubules (1 μM) as indicated.

Figure 4. CENP-E, BubR1, and microtubules form a ternary complex.

(A and B) After centrifugation through glycerol cushions, BubR1/CENP-E/GMPCPP microtubules complex formation was assayed by Immunoblot (A) or triple immunofluorescence for BubR1, CENP-E, along with microtubules (B).

(C) Silencing the mitotic checkpoint signaling upon microtubule capture by CENP-E. CENP-E activates BubR1 kinase activity, resulting in Mad2 recruitment onto unattached kinetochores and mitotic checkpoint activation (top panel). Spindle microtubule capture by CENP-E inhibits the CENP-E-dependent BubR1 kinase activity without dissociation from it, prevents kinetochore recruitment of Mad2, thus, silences the mitotic checkpoint signaling (bottom panel).

Supplemental Figure 1. A requirement of the N-terminal microtubule motor domain for CENP-E in metaphase chromosome alignment.

(A) Purification of recombinant Xenopus CENP-E_{tail}. Initial E. coli lysates encoding 6His-CENP-E_{tail} (lane 1) and recombinant protein (arrow head) after purification over Ni-NTA agrose beads (Qiagen) by standard methods (lane 2).

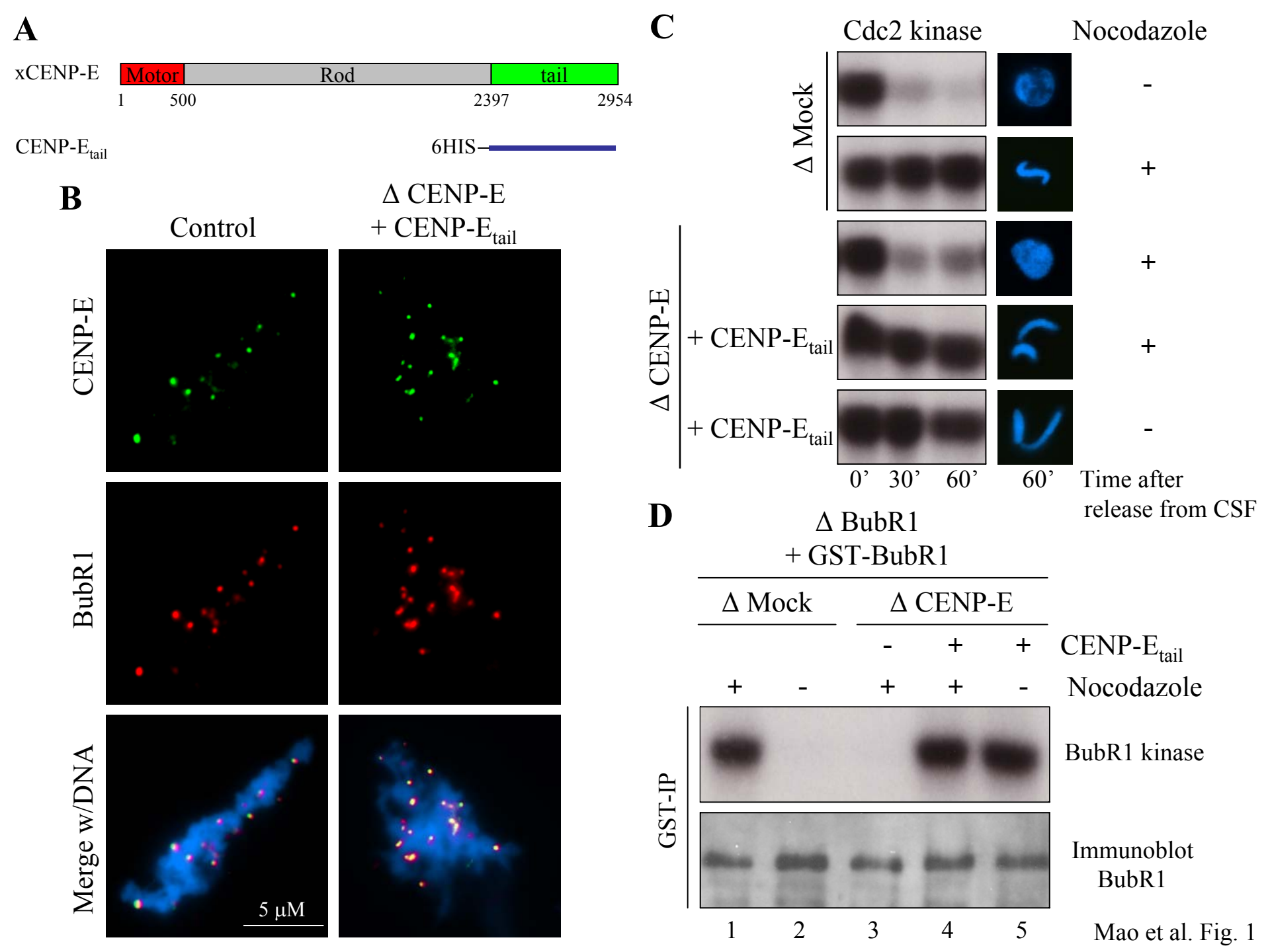
(B) Representative structures formed in control (left panel), or CENP-E depleted and supplemented with CENP-E_{tail} (right panel) egg extracts. Tubulin is shown in red; chromosomes, in blue.

(C) Quantification of structures formed from sperm nuclei in control extracts (n = 26), CENP-E depleted extracts and supplemented with CENP-E_{tail} (n = 25). Data are

presented from one representative experiment. Structure were scored as bipolar spindles with chromatin aligned at the metaphase plate, bipolar spindles with misaligned chromosomes, and other, including monopolar spindles, multipolar structures, and groups of chromosomes apparently unassociated with microtubules.

Supplemental Figure 2. Microtubule capture by CENP-E is required for preventing Mad2 kinetochore recruitment.

Immunofluorescence detection of Mad2 (top row) and BubR1 (center row) in CENP-E-depleted CSF egg extracts which supplemented with recombinant CENP-E (left panel) or CENP-E_{tail} (right two panels) with spindle microtubules (in the absence of nocodazole) or not as indicated after addition of sperm nuclei for 60 min.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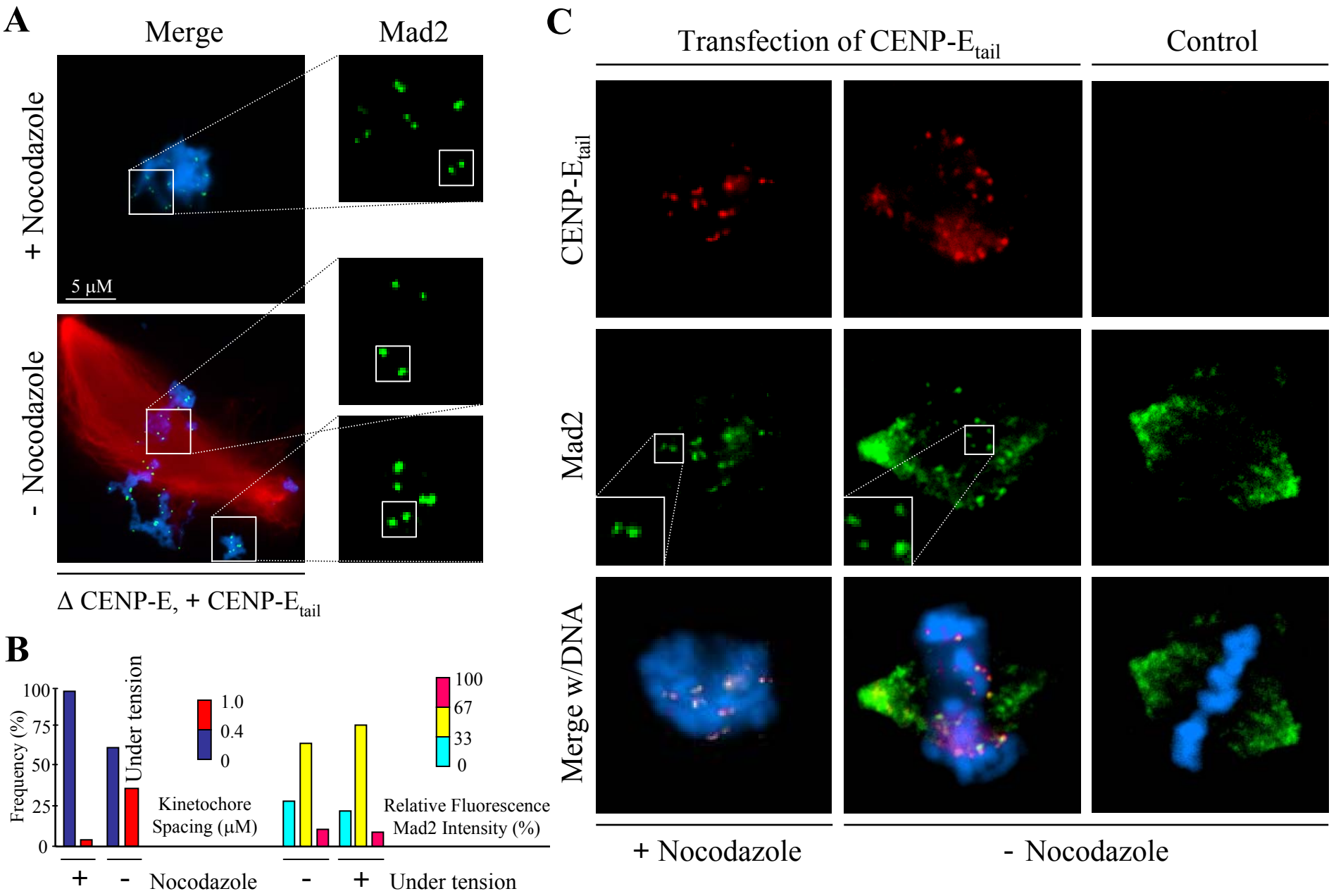

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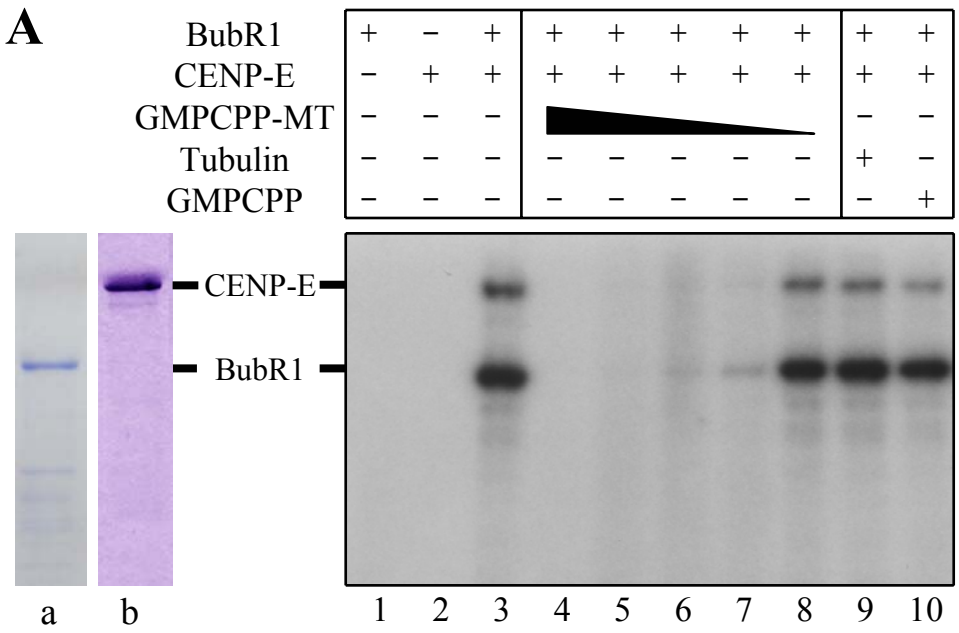

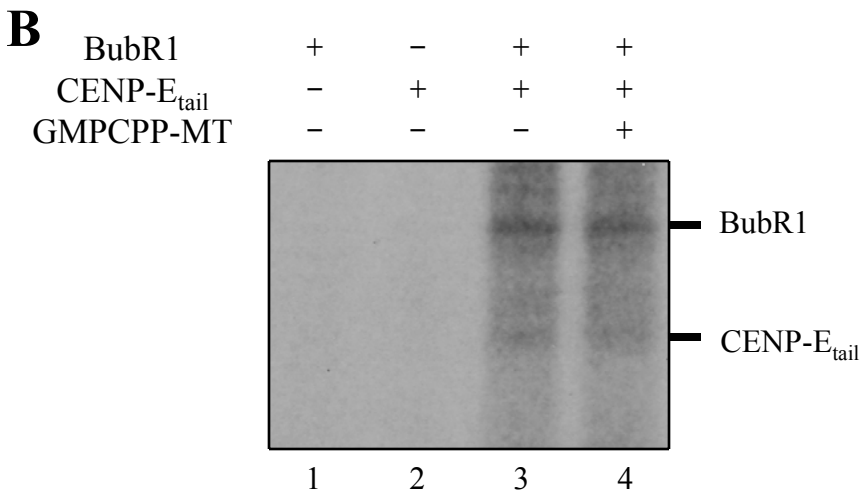

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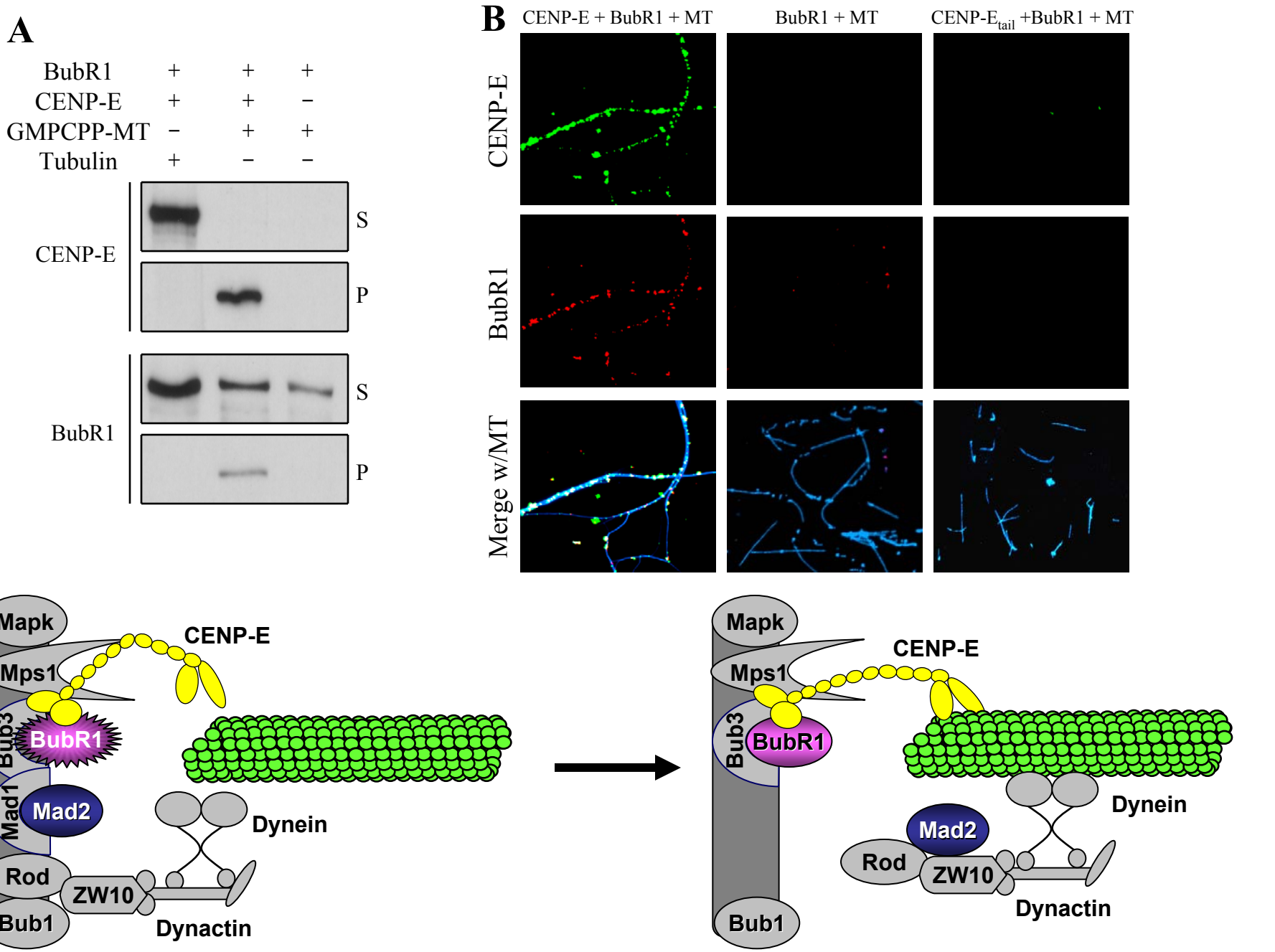

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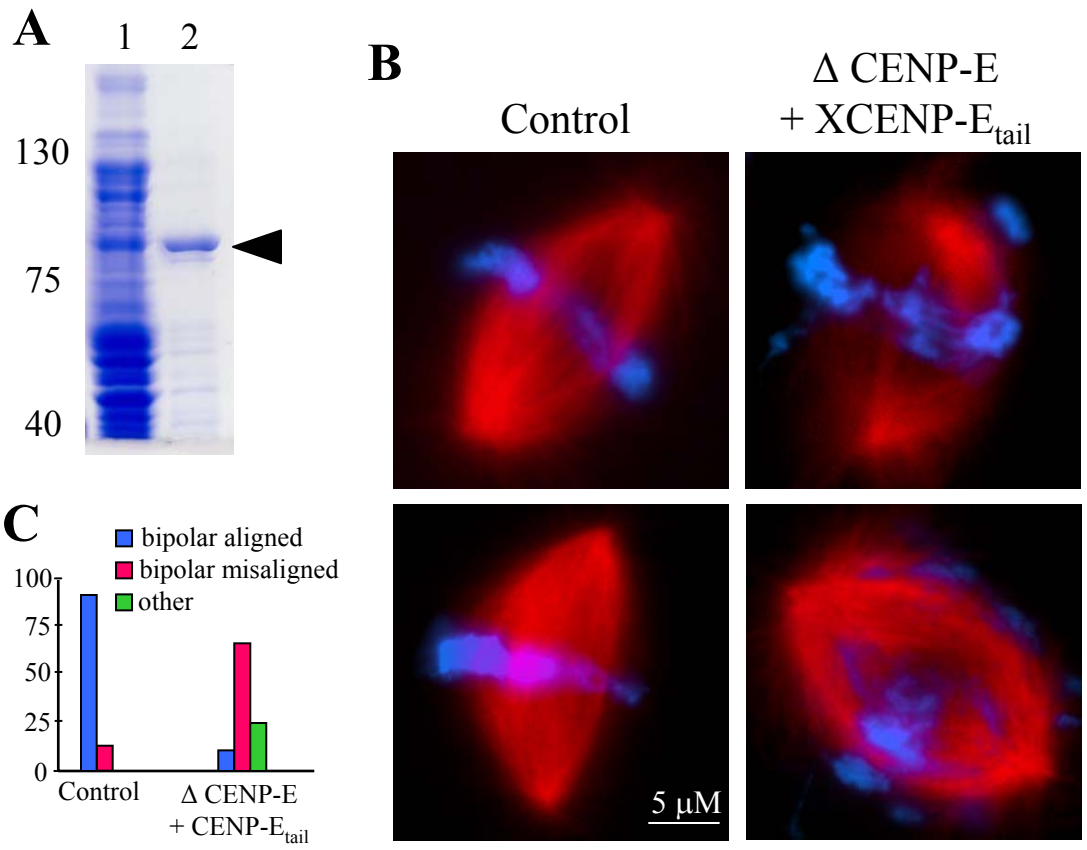

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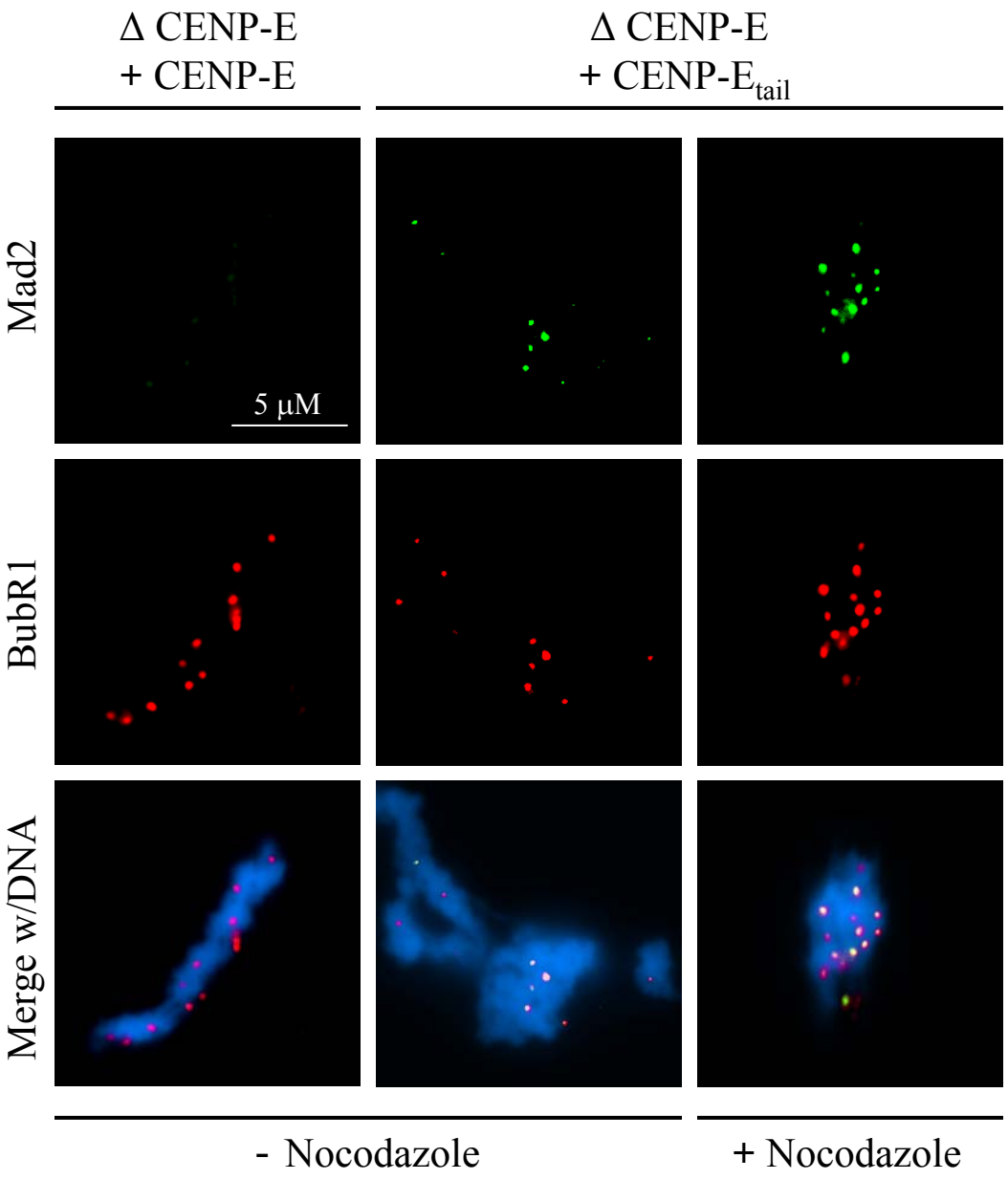

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