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A Rae1-Containing Ribonucleoprotein Complex Is Required for Mitotic Spindle Assembly

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Summary

Centrosome-independent microtubule polymerization around chromosomes has been shown to require a local gradient of RanGTP, which discharges mitotic cargoes from the nuclear import receptor importin β. Here, we have used an activity-based assay in *Xenopus* egg extracts to purify the mRNA export protein Rae1 as a spindle assembly factor regulated by this pathway. Rae1 is a microtubule-associated protein that binds directly to importin β. Depletion of Rae1 from extracts or cells severely inhibits mitotic spindle assembly. A purified Rae1 complex stabilizes microtubules in egg extracts in a RanGTP/importin β-regulated manner. Interestingly, Rae1 exists in a large ribonucleoprotein complex, which requires RNA for its activity to control microtubule dynamics in vitro. Furthermore, we provide evidence that RNA associates with the mitotic spindle and that it plays a direct, translation-independent role in spindle assembly. Our studies reveal an unexpected function for RNA in spindle morphogenesis.

Introduction

Proper segregation of chromosomes during cell division is mediated by the mitotic spindle apparatus, a complex and dynamic macromolecular machine composed of polymerized tubulin and many interacting proteins. Bipolar spindle assembly requires the selective local stabilization and organization of microtubules. This can be achieved by at least two partially redundant pathways. In most somatic cells, microtubule organization is strongly influenced by centrosomes. The centrosome duplicates prior to the onset of mitosis, generating two foci that nucleate microtubules, which then interact with chromosomes through a "search and capture" mechanism (Kirschner and Mitchison, 1986). However, bipolar spindles can also assemble in the absence of centrosomes (Karsenti and Vernos, 2001). In this pathway, microtubules are nucleated and stabilized around chromosomes and subsequently "self-organize" to form a mitotic spindle in the absence of paired microtubule nucleating sites. Chromatin-induced spindle assembly is most dramatically illustrated by the ability of plasmid DNA coated beads to organize bipolar spindles in *Xenopus* egg extracts (Heald et al., 1996). Although chromatin-stimulated mechanisms are most obvious in female meiotic cells, studies inactivating centrosomes through mutation or ablation in other cell types suggest that this pathway is a general feature of spindle assembly (Budde and Heald, 2003).

Chromatin-induced spindle assembly appears to depend on the action of the small GTPase Ran. In *Xenopus* egg extracts, high RanGTP concentrations are sufficient to induce spontaneous polymerization of microtubules, which then organize into aster- and spindle-like structures in the absence of centrosomes or chromosomes (Kahana and Cleveland, 1999). The chromatin bound RanGEF, RCC1, produces RanGTP in the vicinity of chromosomes, and it was proposed that RanGTP is responsible for the local release of spindle-promoting factors from the nuclear transport receptor importin β (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). Once discharged, these spindle-promoting factors are thought to be activated to promote microtubule stabilization and organization in the vicinity of chromosomes. Although several RanGTP- and importin β-regulated spindle-promoting factors have now been identified (Harel and Forbes, 2004), very little mechanistic data is available for how importin β can act as a repressor of their activities. In addition to the RanGTP pathway, chromatin-induced spindle assembly was also recently shown to be regulated by the chromosomal passenger complex containing the Aurora B kinase (Sampath et al., 2004). Despite these advances, it is still poorly understood how chromatin mediates spindle assembly and contributes to spindle function in somatic cells.

To date, all factors identified downstream of Ran in the chromatin microtubule pathway in mitosis bind to importin β indirectly through importin α and thus represent only a subset of importin β binding proteins. We have previously obtained evidence for additional Ran-regulated spindle-assembly factors that function independently of importin α (Nachury et al., 2001). To identify these factors, we have utilized an activity-based assay and purified the mRNA export factor Rae1 as an important Ran-regulated activity involved in spindle formation. We show that Rae1 binds to microtubules in vivo and in vitro and is required for both Ran aster and sperm spindle assembly in vitro. Surprisingly, Rae1 is part of a ribonucleoprotein (RNP) complex, and the Rae1 spindle-promoting activity requires RNA. Furthermore, we provide evidence that correct spindle formation in *Xenopus* egg extracts is RNA dependent, suggesting that RNA plays a general, translation-independent role in mitotic spindle assembly.

Results

Identification of Rae1 as a Component of a Ran-Regulated Aster-Promoting Activity

Our previous studies have demonstrated the existence of multiple activities that function downstream of

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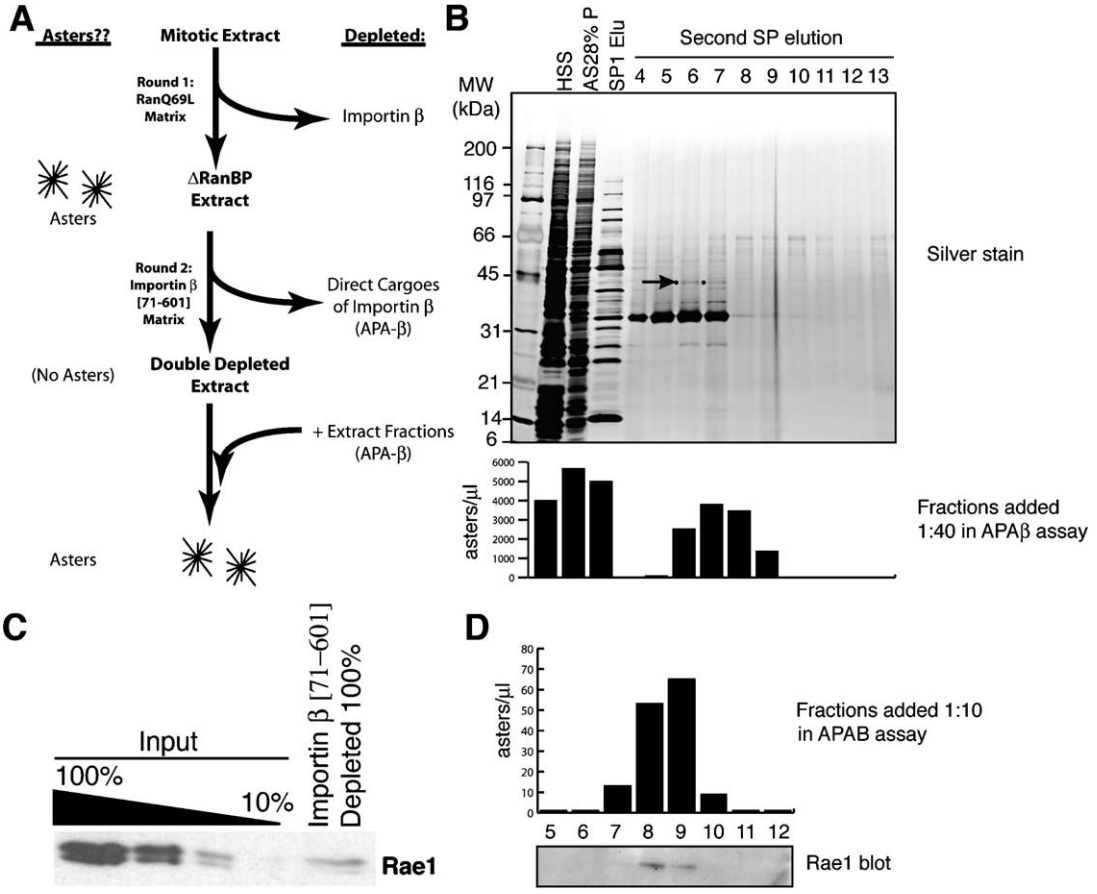

Figure 1. Rae1 Is a Ran-Regulated Aster-Promoting Activity

(A) Schematic of the assay used to identify Rae1. Sequential affinity chromatography steps were used to deplete metaphase-arrested CSF *Xenopus* egg extracts: first, a RanGTP matrix was used to remove RanGTP binding proteins including importin β (ΔRanBP Extract), freeing cargoes that caused spontaneous microtubule aster formation. Second, an importin β matrix was used to deplete direct binding cargoes (Double Depleted Extract), abolishing the asters. Rescue of the aster-promoting activity (APA) in the double-depleted extract was then assayed following the addition of diluted CSF extract subjected to fractionation steps (for details of the purification scheme see also Figures S1 and S2).

(B) Silver-stained gel of final SP column fractions from a purification of APA-β. Arrow indicates the position of Rae1, which was identified by mass spectrometry. Graph represents the aster-forming activity of each column fraction during the purification. The major band at ~35 kDa is a contaminant that was not consistently present in the purification.

(C) Western blot of double-depleted extract showing that ~75% of Rae1 is removed by this treatment compared to a dilution series of the ΔRanBP Extract (100%, 50%, 25%, and 10%).

(D) Western blot of Rae1 in the final SP column fractions from a different purification indicates that Rae1 cofractionates with the peak of the aster-forming activity in the final purification step.

RanGTP and importin β in spindle assembly in *Xenopus* egg extracts (Nachury et al., 2001). One activity was independent of importin α and is therefore distinct from previously identified factors such as NuMA and TPX2 (see Figure S1 in the Supplemental Data available with this article online). We have termed this novel activity aster-promoting activity β (APA-β). In order to characterize this importin α-independent activity, we devised an assay using meiotic cytostatic factor-arrested (CSF) extracts depleted of both importin β and its directly bound cargoes using sequential affinity chromatography steps (Figure 1A). In the first step, a RanGTP matrix was used to deplete importin β, leaving its cargoes behind. Spontaneous asters formed in these metaphase extracts, presumably due to the liberation of mitotic cargoes that promote microtubule polymerization and organization. In the next step, the extract was treated with a matrix containing a C-terminal truncation of importin β, importin β [71–601], which lacks the importin α interaction domain (Figure 1A). Aster assembly was abolished in these extracts and could not be rescued by addition NuMA or TPX2, providing evidence for the existence of other essential mitotic cargoes of importin β (Figure S1). To identify novel cargoes, we used the extracts depleted of importin β and its importin α-independent cargoes in an assay to biochemically purify an APA-β activity that restored microtubule polymerization and aster formation (Figure S2). A polypeptide with an apparent molecular weight of ~40 kDa (Figure 1B, arrow) consistently cofractionated with the activity over

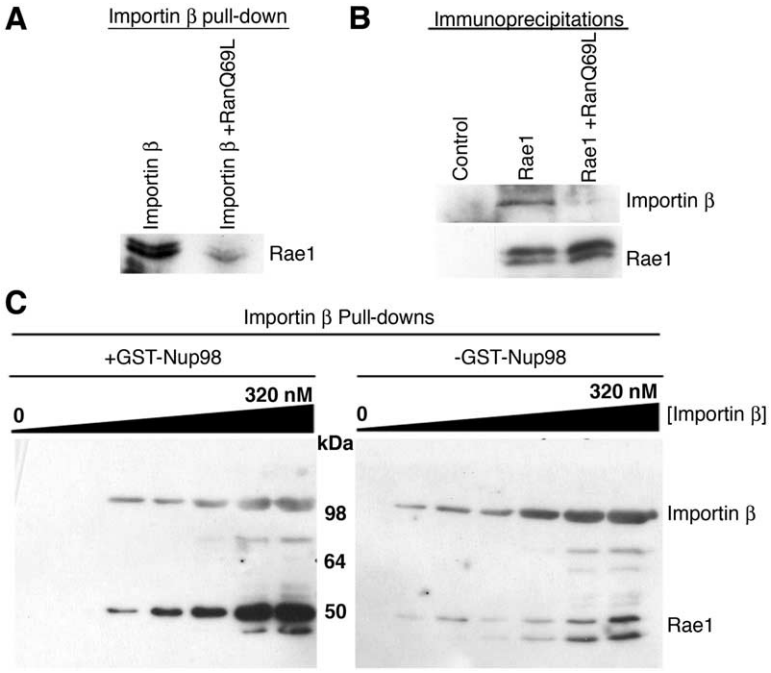

Figure 2. Rae1 Binds to Importin β in Extracts and In Vitro

(A) Pull-downs using recombinant importin β from CSF extracts (±25 μM RanQ69L) probed for Rae1.

(B) Immunoprecipitation using anti-Rae1 antibodies (or nonspecific IgG) from CSF extracts (±25 μM RanQ69L) probed for Rae1 and importin β.

(C) In vitro pull-downs using increasing concentrations of recombinant importin β from a solution containing pure recombinant Rae1 (±recombinant GST-Nup98). Rae1 and importin β are detected by Western blot with anti-Rae1 antibodies, which also recognize the N-terminal 6-HIS tag present on importin β.

several chromatographic steps in different trials of the purification procedure. This polypeptide was identified as the mRNA export factor Rae1 by tandem quadrupole ion trap mass spectrometry. Western blot analysis of different steps in the assay and purification procedure revealed that ~70% of Rae1 was depleted from extracts by importin β [71–601] (Figure 1C) and confirmed that Rae1 cofractionated with the peak of the aster promoting activity (Figure 1D). Therefore, we chose to investigate whether Rae1 is a novel Ran-regulated factor important for spindle assembly.

Rae1 Binds Directly to Importin β, and This Interaction Is Enhanced by Nup98

The activity of Rae1 in our aster formation assay suggested that Rae1 binds to importin β independently of importin α and that RanGTP should inhibit this interaction. To investigate these predictions, we performed pull-down experiments from CSF extracts using S-tagged recombinant importin β proteins. Rae1 bound specifically to full-length importin β and to importin β [71–601] (Figure 2A and data not shown). The binding of Rae1 to importin β was RanGTP sensitive, as addition of 25 μM RanQ69L-GTP (a mutant form of Ran-deficient in GTP hydrolysis) abolished the interaction (Figure 2A). We also performed the reciprocal experiment using Rae1 antibodies to immunoprecipitate Rae1 and probed for the presence of importin β. Western blots confirmed that importin β coimmunoprecipitated with Rae1 and that this interaction was inhibited by RanQ69L-GTP (Figure 2B).

To determine whether Rae1 bound to importin β directly or whether the interaction required additional factors, we used pure recombinant proteins in an in vitro binding assay. Although importin β reproducibly bound to Rae1, their interaction was weak (Figure 2C, right panel), suggesting that additional proteins may facilitate the binding of Rae1 to importin β in the extracts. Nup98 was a good candidate, as it was previously shown to bind tightly to both Rae1 and importin β (Blevins et al., 2003; Fontoura et al., 2000). Addition of recombinant GST-Nup98 to the in vitro interaction assays increased the levels of binding of importin β to Rae1 by ~10-fold (Figure 2C, left panel). These results demonstrate that Rae1 is an importin α-independent mitotic cargo of importin β and that the interaction between these proteins is enhanced by Nup98.

Rae1 Localizes to Sperm Spindles and Ran Asters and Binds Directly to Microtubules In Vitro

To begin characterizing its function, we localized Rae1 in *Xenopus* egg extracts by immunofluorescence using specific Rae1 antibodies that were raised against the recombinant protein. Consistent with a previous report (Blevins et al., 2003), Rae1 was concentrated in interphase nuclei (data not shown). In mitotic extracts, Rae1 was present on spindles that formed around demembranated sperm nuclei and on RanGTP-induced microtubule asters (Figures 3A and 3B). Rae1 was enriched at spindle poles and at the focus of Ran asters but was also present along the entire length of microtubules in these structures. Furthermore, Rae1 was localized in a punctate pattern on sperm chromosomes. Four distinct Rae1 antibodies gave identical staining patterns, and addition of Oregon green-labeled recombinant Rae1 to extracts revealed a similar localization (data not shown), indicating that these data accurately reflect the distribution of Rae1. The Rae1 chromatin staining did not require microtubules, as the Rae1 punctae remained on mitotic chromosomes following nocodazole treatment (data not shown).

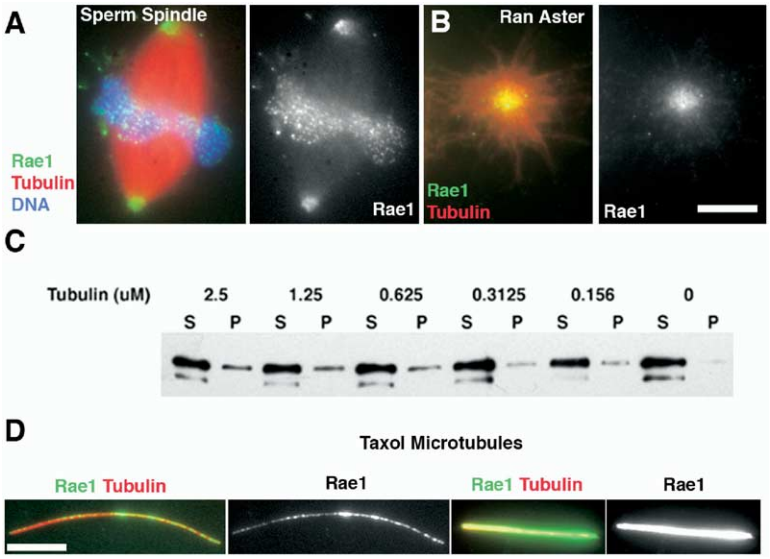

Figure 3. Rae1 Binds to Microtubules in Extracts and In Vitro

(A and B) Indirect immunofluorescence localization of Rae1 in a mitotic spindle (A) and a RanGTP-induced microtubule aster (B) assembled in CSF extracts. Rae1 is pseudocolored in green; microtubules are red, and DNA is blue.

(C) Recombinant Rae1 binding to increasing concentrations of taxol-stabilized microtubules in a pelleting assay, detected by Western blot. S, Supernatant. P, Pelleted microtubules.

(D) Indirect immunofluorescence localization of recombinant Rae1 on an individual taxol-stabilized microtubule (left) and a microtubule bundle (right).

Scale bar is 10 μm.

The localization of Rae1 in sperm spindle- and Ran aster-assembly reactions suggested that Rae1 was binding to microtubules. To test whether Rae1 binds directly to microtubules in vitro, recombinant Rae1 was incubated with increasing concentrations of purified, taxol-stabilized microtubules, which were pelleted and analyzed for the presence of Rae1 (Figure 3C). Rae1 bound directly to microtubules with a low affinity. Microtubule binding appeared to be specific, as it was not affected by the presence of 10 mg/ml BSA but was blocked by preincubation of recombinant Rae1 with Rae1 antibodies (data not shown). Immunofluorescence analysis of pelleted microtubules demonstrated that Rae1 was not enriched at either end but bound along the entire length of microtubules (Figure 3D). Incubation of recombinant Rae1 with 500-fold molar excess of importin β and Nup98 did not alter Rae1 microtubule binding, indicating that importin β does not regulate the interaction of Rae1 with microtubules (data not shown).

Rae1 Is Required for Spindle Assembly in Extracts and in Human Cells

To analyze the role of Rae1 in spindle assembly, we used anti-Rae1 antibodies to deplete Rae1 from extracts and assayed the effects on both spindle assembly in the presence of sperm nuclei and on microtubule aster formation in the presence of RanGTP. We were able to deplete 60%–70% of Rae1 from the extracts as compared to mock-depleted controls (Figure 4A). Depletion of Rae1 decreased the number of Ran asters to ~30% of control reactions and resulted in the appearance of long bundled microtubules, which were not organized into focused asters (Figure 4F). Aster formation could be partially restored by the addition of recombinant Rae1 (Figure 4B) to the endogenous level of ~100 nM (Figure 4C; n = 8 experiments, p = 0.01), suggesting that Rae1 is required for Ran aster assembly (Figures 4H and 4J). A complete rescue could be achieved by adding back the peak APA-β/Rae1-containing fraction from our purification to the Rae1-depleted extracts (Figure 4J; n = 4 experiments). This result suggested that Rae1 by itself has only limited activity and requires association with other factors in the extract to promote aster formation. This is consistent with the observation that recombinant Rae1 alone had no APA-β activity in the double-depleted extracts (data not shown). We also found that depletion of Rae1 severely inhibited the assembly of spindle structures around sperm nuclei in CSF extracts (Figures 4E and 4G). Similar to the effect on Ran asters, Rae1 depletion resulted in sparse, long bundled microtubules, which did not organize into a spindle. Although we did not attempt to rescue this defect with the APA-β peak fraction, due to limited quantities of this fraction, the addition of recombinant Rae1 partially restored this spindle assembly (n = 4 experiments; p = 0.05; Figures 4I and 4K). We conclude that Rae1 is a microtubule binding protein necessary for the assembly of sperm spindles and Ran asters in *Xenopus* egg extracts.

To test whether Rae1 has a similar function in spindle assembly in somatic cells, we performed RNA interference experiments in human tissue culture cells. siRNAs directed against human Rae1 were transfected into HeLa cells, and the loss of Rae1 protein was analyzed by Western blot (Figure 5A) and immunofluorescence (Figures 5B and 5D). After 72 hr, Rae1 protein levels were reduced by ~80% (Figure 5A), and a more than 2-fold increase in the mitotic index was observed in the siRNA-treated cells compared to controls (6.9 ± 2.5% for Rae1siRNA versus 3 ± 0.2% for control; n = 3 experiments). In ~55% of mitotic cells, Rae1 depletion resulted in defects in spindle assembly, chromosome alignment, or segregation. Chromosomes failed to align properly in prometaphase/metaphase (Figure 5G), and a significant increase in the appearance of multipolar spindles (~30% of mitotic cells Figures 5D, 5F, and 5I) and massive anaphase segregation defects (Figure 5E) were observed. Staining cells for the centrosomal protein pericentrin revealed a large increase in the number

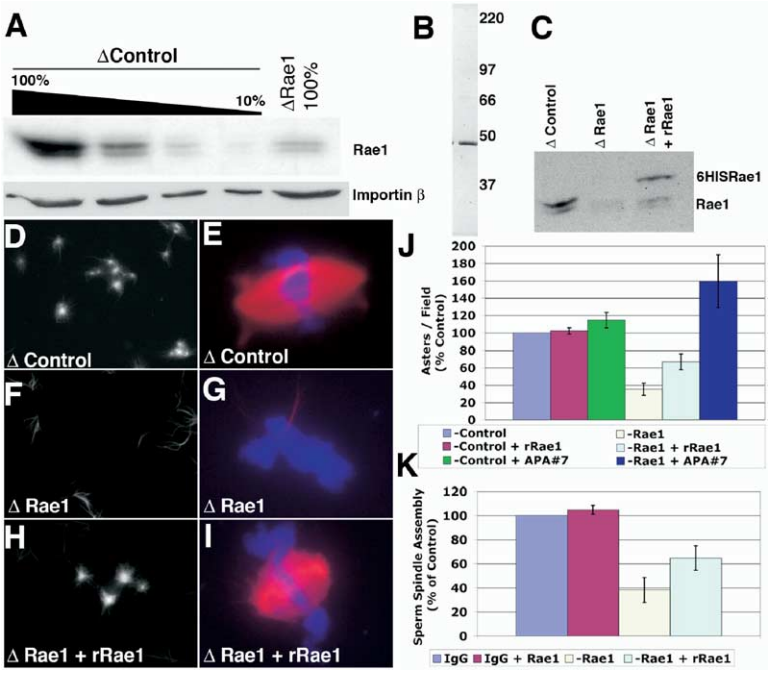

Figure 4. Rae1 Is Required for Ran Aster and Sperm-Spindle Assembly

(A) Rae1 was depleted from CSF extracts using antibody 1926 or 2238 and compared to a dilution series (100%, 50%, 25%, and 10%) of mock-depleted extract, with importin β as a loading control.

(B) Purity of recombinant Rae1 (rRae1) shown by SDS-PAGE and Coomassie staining.

(C) Western blot with anti-Rae1 antibodies on control, Rae1-depleted, and Rae1-depleted + rRae1 extracts demonstrating that Rae1 was added back to approximately the endogenous concentration.

(D, F, and H) RanGTP-induced microtubule asters formed in control, Rae1-depleted, and Rae1-depleted + rRae1 extracts.

(E, G, and I) Sperm spindles assembled in control, Rae1-depleted, and Rae1-depleted + rRae1 extracts. Microtubules are red and DNA is blue.

(J) Quantification of RanGTP-induced microtubules asters/field, comparing Rae1-depleted and mock-depleted extracts (±rRae1 or APA-β peak fraction). n = 8 independent experiments; error bars are the SEM.

(K) Quantification of sperm nuclei that induced spindle assembly, comparing Rae1- and mock-depleted extracts (±rRae1). n = 4 independent experiments; error bars are the SEM.

of pericentrin foci in Rae1 siRNA-treated mitotic cells (Figures 5H, 5I, and 5K). The extra spindle poles seemed to be functional, as they appeared to pull chromosomes away from the main spindle (Figures 5F and 5G), contributing to serious chromosome-alignment defects. Thus, Rae1 is required for proper spindle assembly and chromosome segregation in human somatic cells, as well as in *Xenopus* egg extracts.

Rae1 Functions in an RNP Complex to Nucleate/Stabilize Microtubules

Our observations that Rae1 is a microtubule binding protein required for proper spindle assembly suggested a potential direct role for Rae1 in the regulation of spindle microtubule dynamics and/or organization. However, we found that recombinant Rae1 did not possess microtubule nucleating, stabilizing, or bundling activity when assayed with pure tubulin (data not shown). We therefore hypothesized that Rae1 requires additional factors and/or posttranslational modifications for activity. To test whether native Rae1 in association with other factors possessed microtubule nucleation or stabilization activity, we immunopurified endogenous Rae1 from an ammonium sulfate pellet of a high speed *Xenopus* extract (step 1, Figure S2) using Rae1 antibody-coated beads. Upon isolation, beads were washed and then reintroduced into double-depleted extracts from which direct binding importin β cargoes had been removed and therefore lacked APA-β activity (Figure 1). As shown in Figure 6B, Rae1 beads specifically induced the nucleation/stabilization of microtubules in these extracts (Figure 6B). In contrast, IgG-coated control beads did not have any microtubule nucleation or stabilization activity (Figures 6A and 6E). These results show that Rae1 in its native form is able to rescue microtubule nucleation/stabilization in the double-depleted extract and suggest that Rae1 requires additional cellular cofactors for its function. Importantly, these data also demonstrate that the APA-β activity and the Rae1-associated activity are functionally interchangeable (see also Figure 4).

To determine if the activity of the Rae1 beads was regulated by importin β, we incubated the beads with recombinant importin β [71–876] or [71–601], washed them extensively, and then reintroduced them into double-depleted extract. Importin β treatment severely diminished the ability of the Rae1 beads to nucleate or stabilize microtubules (Figures 6C and 6E), demonstrating that the native Rae1 complex is a target of importin β regulation.

To identify Rae1-associated protein(s) that could contribute to the observed microtubule nucleation/stabilization activity, we analyzed the composition of the purified Rae1 complex. Immunopurifications using two different antibodies demonstrated that Rae1 specifically associates with more than ten polypeptides in CSF extracts (Figure 6F). The major Rae1-associated proteins were subjected to MALDI mass spectrometry after trypsin digestion, leading to the identification of importin β, Nup98, Maskin, Fubp2, Rap55, Vera, CPSF25, and CBTF^{122} (Figure 6F). Whereas Nup98 is a known Rae1 binding protein (Figure 2; Blevins et al., 2003), Maskin, Fubp2, Rap55, Vera, CPSF25, and CBTF^{122} are all mRNA-associated proteins that have been implicated in various steps of mRNA biogenesis (Deshler et al., 1997; Groisman et al., 2000; Kroll et al., 2002; Lieb et al., 1998). Interestingly, two of these pro-

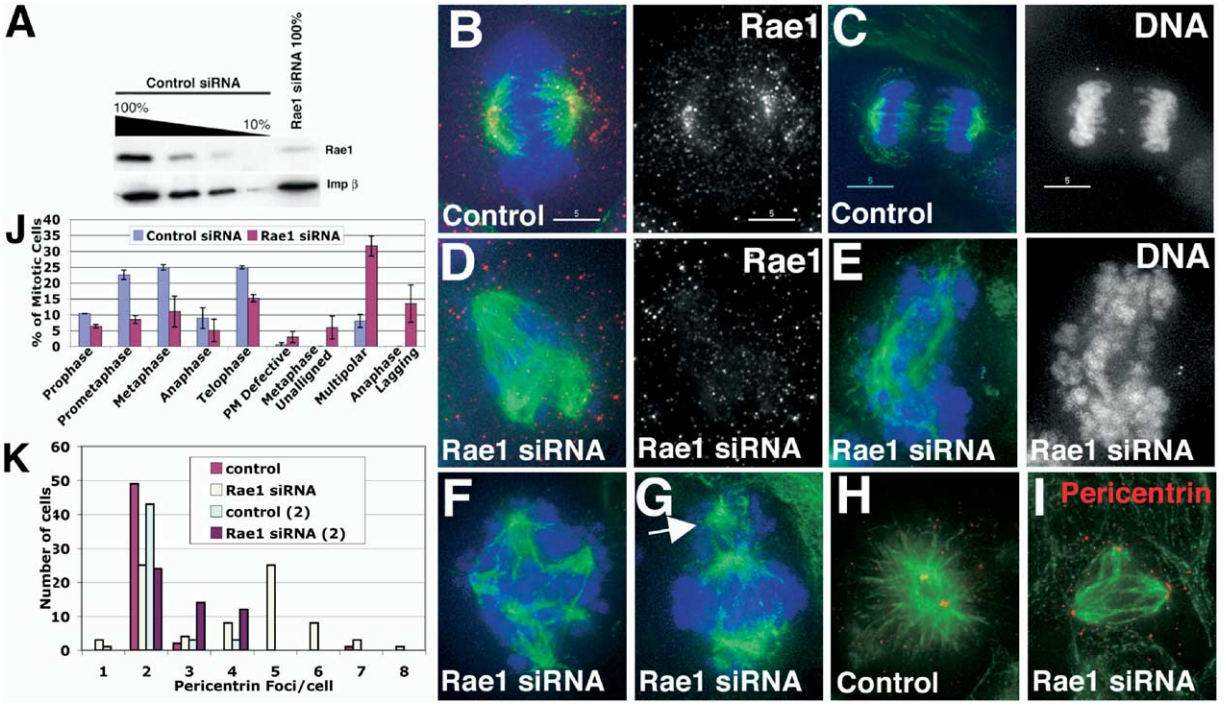

Figure 5. Rae1 Is Required for Spindle Assembly and Function in HeLa Cells

(A) Western blot of Rae1 and importin β (as a loading control) comparing Rae1 siRNA-treated cells to a dilution series (100%, 50%, 25%, 10%) of control siRNA-treated cells.

(B–I) Fluorescence images of Rae1 siRNA-treated and control cells. Microtubules are pseudocolored in green; DNA is blue, and Rae1 or pericentrin is red.

(B and C) Control metaphase and anaphase cells.

(D) Rae1 siRNA-treated cell exhibiting a tripolar spindle with reduced Rae1 staining at the poles compared to the control cell.

(E–G) Rae1 siRNA-treated cells undergo aberrant anaphase with many lagging chromosomes (E) and are often multipolar with defects in chromosome alignment (D, F, and G). Extra spindle poles can sequester chromosomes away from the main spindle ([G], arrow).

(H and I) Control and Rae1 siRNA-treated cells stained for pericentrin to mark spindle poles/centrosomes. The Rae1 siRNA-treated cell exhibits three pericentrin foci.

(J) Quantification of the mitotic defects observed upon Rae1 depletion by siRNA, showing a reduction in normal mitotic figures and an increase in defective prometaphase, metaphase, and anaphase and a high percentage of multipolar spindles. n = 3 independent experiments; error bars are the SD.

(K) Quantification of the number of pericentrin foci/mitotic cell in control and Rae1 siRNA-treated cells. Results from two independent experiments are shown.

Scale bars are 5 μm.

teins, Maskin and Fubp2, were previously shown to function in mRNA localization in a microtubule-dependent manner (Groisman et al., 2000; Kroll et al., 2002), suggesting that Rae1 might provide a link between mRNA localization and spindle assembly.

The association of Rae1 with multiple mRNA binding proteins implied that Rae1 might be part of a large RNP complex. We therefore examined whether the purified complex contained RNA or required RNA for its function. The immunoprecipitated Rae1 complex contained a wide size range of RNAs as assayed by agarose gel electrophoresis, while control IgG or Eg5 IPs contained little or no associated RNA (Figure 6G). To directly test whether RNA is necessary for the activity of the Rae1 complex, the ammonium sulfate fraction was treated with RNaseA prior to Rae1 immunoprecipitation, and then Rae1 beads were analyzed for their activity in double-depleted extracts. Surprisingly, we found that the ability of Rae1 beads to nucleate/stabilize microtubules was diminished after RNaseA treatment whereas mock treatment had no effect (Figures 6D and 6E). The loss of activity coincided with the reduction in binding of at least four polypeptides to the complex (Figure 6F), suggesting that these polypetides interact with Rae1 in an RNA-dependent manner. Although we were unable to test the effect of RNase treatment on the APA-β peak fractions in the APA-β assay, we found that RNase treatment significantly reduced the APA activity of the ammonium sulfate pellet fraction in the APA-β assay (Figure S3), demonstrating that a component of the APA-β activity is RNase sensitive. Taken together, these results show that the spindle-associated protein Rae1 is present in an RNP and that the Rae1-RNP requires RNA for its activity or complex integrity.

RNAs Are Required for Spindle Assembly in Egg Extracts

The results described above indicated that RNA might be required for spindle assembly in egg extracts. To

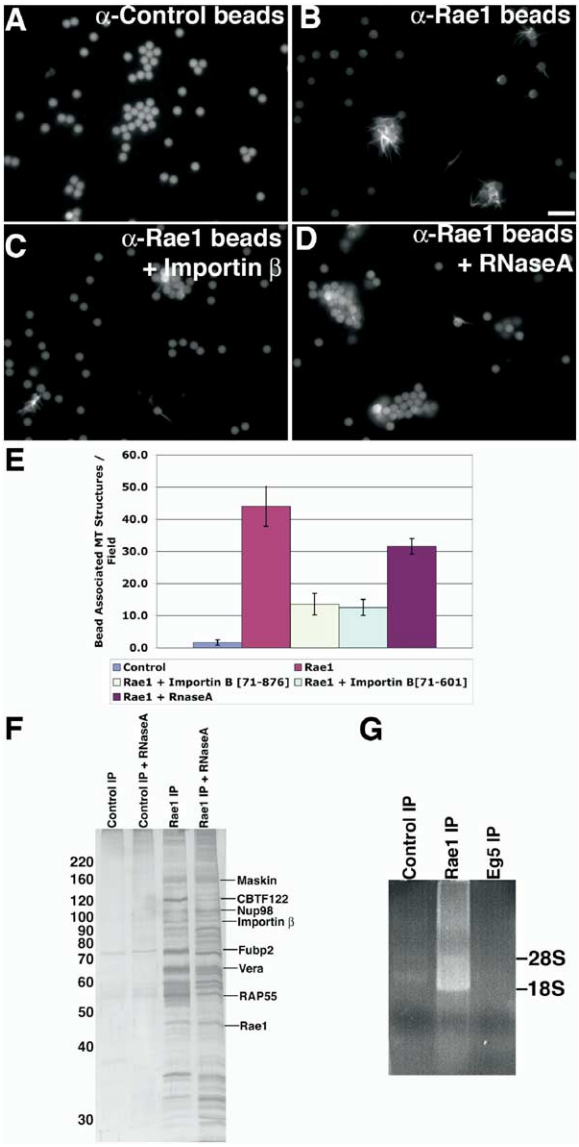

Figure 6. Anti-Rae1 Beads Nucleate/Stabilize Microtubules in Double-Depleted Extracts

(A and B) Fluorescence images showing that control antibody beads have no activity, while Rae1 antibody beads induce polymerization of abundant microtubules both from large bead clusters and from individual beads. Microtubules are visualized by the incorporation of rhodamine-labeled tubulin.

(C) Anti-Rae1 beads incubated with recombinant importin β [71–876] exhibit dramatically reduced microtubule nucleation capacity.

(D) Anti-Rae1 beads from an RNaseA-treated extract show a reduced number of microtubules associated with large bead clusters.

(E) Quantification of the number of microtubules nucleated by individual beads and bead clusters (>3 beads) for the conditions described. n = 4 independent experiments. Scale bar is 10 μm; error bars are the SEM.

(F) Proteins associated with IgG and Rae1 beads (±RNaseA) analyzed by SDS-PAGE. Rae1-interacting proteins identified by mass spectrometry are indicated to the right of the gel.

(G) RNA composition of eluates from IgG, anti-Rae1, and anti-Eg5 beads analyzed by agarose gel electrophoresis and ethidium bromide staining. Positions of 18S and 28S ribosomal RNAs are marked.

test this, we treated CSF-arrested extracts with RNaseA to digest all RNAs present in the extract (Figure 7F) and examined the effects on sperm spindle and Ran aster assembly. We found that addition of RNase severely inhibited sperm spindle and Ran aster assembly, resulting in many long microtubules that were not focused into Ran asters or spindle poles (Figure 7). This phenotype was similar to what was observed upon Rae1 depletion but much more severe. The effect of RNase treatment was dependent on the catalytic activity of RNase, as the addition of S protein (a proteolytic cleavage product of RNaseA that has a dramatically reduced catalytic activity) or the S peptide fragment alone caused no defects in Ran aster or sperm spindle assembly, while addition of S peptide plus S protein (which recreates a catalytically active RNase) to the extract phenocopied RNaseA addition (Figure 7H). Furthermore, the RNase phenotype was not an artifact of the presence of high concentrations of RNase in the extract, as RNase immobilized on Sepharose beads that could be removed prior to the spindle assembly reaction produced an identical phenotype as direct RNase addition to extract (Figure 7H). The effects of RNase on microtubule organization appeared specific, since no significant defect in interphase chromatin morphology, nuclear formation, chromatin condensation, or kinetochore assembly and no change in the profile of MAPs was observed in the RNase-treated extracts (Figures S4–S7). Together, these results suggest that RNAs or intact RNPs are required for correct Ran aster and spindle assembly in egg extracts.

Several previous studies have suggested that regulation of local translation of specific RNAs on the mitotic spindle might play a role in spindle morphogenesis and cell cycle control (Groisman et al., 2000). To determine whether protein translation is required for spindle morphogenesis in *Xenopus* extracts and to test whether the lack of local translation could account for spindle assembly defects observed after RNase treatment, we examined the effects of the translational inhibitors cycloheximide (CHX) and puromycin on Ran aster and sperm spindle assembly. Neither had any significant effect in Ran aster or sperm spindle assembly reactions (Figure 7H and data not shown). This demonstrates that lack of translation cannot account for the spindle-assembly defects observed upon RNase treatment of extracts.

The microtubule arrays present in RNase-treated extracts were somewhat reminiscent of interphase morphology. To determine whether the RNase effect resulted from an altered cell cycle stage or because the extract exited CSF arrest, we measured histone H1 kinase levels in RNase and cycloheximide-treated extracts. Although both RNase and cycloheximide treatment caused a decrease in H1 kinase activity to ~60%–70% of controls, it did not reduce it to interphase levels (Figure 7I). To examine whether reduced H1 kinase activity was responsible for the spindle assembly defects observed in RNase-treated extracts, we restored normal H1 kinase activity by adding the nondegradable cyclin variant, cyclin BΔ90. Whereas addition of cyclin BΔ90 completely restored H1 kinase activity (Figure 7J), no rescue of Ran aster (Figure 7K) or sperm spindle (data not shown) assembly was observed, and the aberrant microtubule morphology was

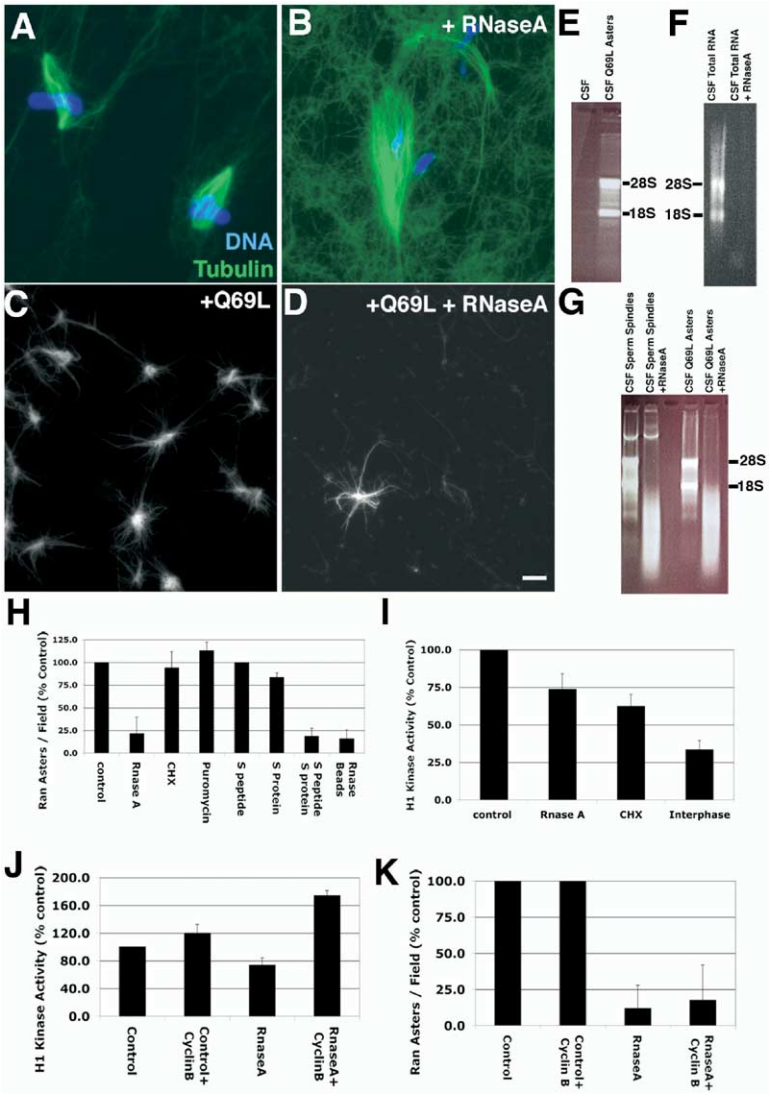

Figure 7. RNA Is Required for Efficient Ran Aster and Sperm-Spindle Assembly

(A–D) Control and RNaseA-treated CSF extracts were combined with sperm nuclei (A and B) or RanQ69L (C and D) to assess spindle and aster formation, respectively. Ran aster assembly was severely inhibited by RNase treatment (D), but a rare Ran aster is shown in (E) to illustrate aster morphology. Microtubules are labeled green and DNA is blue. Scale bar is 10 μm.

(E, F, and G) Extract-, spindle-, and aster-associated RNAs detected by agarose gel electrophoresis and ethidium bromide staining. The positions of the 18S and 28S ribosomal RNAs are indicated.

(E) RNA that pelleted from untreated CSF extracts or RNA that associated with RanQ69L-induced microtubule asters pelleted from CSF extracts.

(F) RNaseA treatment efficiently degrades RNAs in total CSF extract.

(G) RNaseA treatment digests RNAs associated with RanQ69L-induced asters or sperm spindles pelleted from CSF extract.

(H) Degradation of RNA specifically inhibits Ran aster formation, but inhibition of translation does not. Quantification of Ran aster formation in control CSF extracts or extracts treated with RNase; translation inhibitors cycloheximide (CHX) or puromycin; S peptide, S protein, or S peptide + S protein; or RNase-coated beads. n = 5 experiments.

(I) Histone H1 kinase activity in CSF extracts treated with RNase or CHX or in a CSF extract induced to enter interphase. n = 3 experiments.

(J and K) Addition of cyclin BΔ90 elevates histone H1 kinase activity but does not rescue aster defects upon RNase treatment.

(J) H1 kinase activity of control and RNase-treated extracts ±1 μM cyclin BΔ90. n = 3 experiments.

(K) Quantification of Ran aster formation in control or RNase-treated extracts ±1 μM cyclin BΔ90. n = 3 experiments.

In all graphs the error bars are the SD.

not altered. These results strongly suggest that the defects observed upon RNase treatment are not due to a loss of CSF arrest but that intact RNAs or RNPs play a critical translation-independent role in establishing correct spindle morphology.

RNA Associates with the Mitotic Spindle

The RNA requirement for spindle assembly and the presence of Rae1 in an RNP complex prompted us to analyze whether RNAs specifically associate with microtubule structures polymerized by the addition of RanQ69L, taxol, or sperm nuclei to egg extracts. Microtubules were isolated by centrifugation through a glycerol cushion, and associated RNAs were examined by agarose gel electrophoresis (Figures 7E and 7G). Both ribosomal RNAs and RNAs of various sizes were specifically enriched on polymerized microtubules but did not pellet from untreated extracts (Figures 7E and 7G). The nucleic acids present on microtubules were RNAs, as they were degraded by a pretreatment of the extract with RNaseA (Figure 7G).

To visualize the association of RNAs with microtubules, we stained sperm spindles and Ran asters with propidium iodide (PI), a nucleic acid dye that stains both DNA and RNA. We found that PI-stainable, RNase-sensitive material was present specifically on microtubules in both sperm spindles and Ran asters (Figure 8). The RNA was present in granular structures that were enriched around microtubules but did not always perfectly colocalize with microtubules, suggesting that these RNAs may be present in large RNP particles.

Discussion

Rae1 Is a Component of a Ran-Regulated Aster-Promoting Activity

Recent studies have identified several microtubule-associated proteins whose activity is regulated by importin β through importin α (Harel and Forbes, 2004). Here, we have characterized Rae1 as a novel cargo of importin β that functions downstream of RanGTP. We have biochemically purified Rae1 as a component of

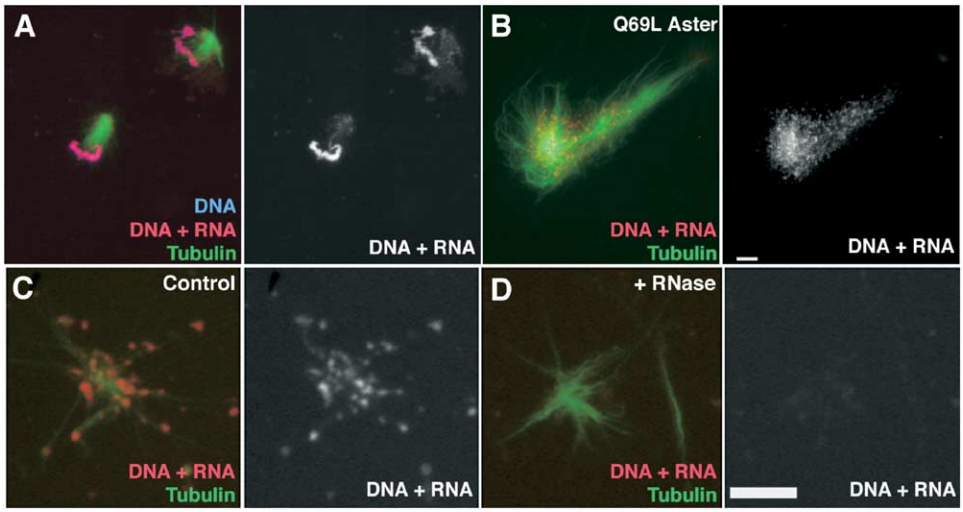

Figure 8. RNA Is Present on Mitotic Microtubules

(A–C) RNA was localized on sperm spindles (A) and Ran asters (B and C) by staining the samples with DAPI (DNA specific dye, blue) and propidium iodide (PI, DNA + RNA dye, red).

(D) Aster-associated PI stainable material is RNase sensitive. A rare Ran aster from an RNase-treated extract showed a decreased amount of granular PI staining. Microtubules are labeled green.

Scale bars are 10 μm.

a microtubule aster-promoting activity (APA-β) that is directly regulated by importin β. However, recombinant Rae1 did not complement aster formation in the double-depleted extract assay, suggesting either that recombinant Rae1 is not fully active or that additional factors are required for full aster formation activity. The latter possibility is supported by the observation that Rae1 is part of a large RNP complex in extracts containing more than ten additional polypetides. Importantly, this complex is able to nucleate microtubules in the double-depleted assay extract, demonstrating that this Rae1-containing complex has APA-β activity. It will be informative to determine which other protein and/or RNA factors in the Rae1 complex are required for full activity and, ultimately, to reconstitute a functionally active Rae1 RNP particle.

As predicted based on its purification assay, the microtubule polymerization stimulating activity of purified Rae1 complex is inhibited by importin β, and we show that recombinant Rae1 binds directly to importin β in vitro. However, this interaction is weak and is greatly stimulated by the addition of the nucleoporin Nup98, which we and others have shown to be a Rae1 binding protein (Blevins et al., 2003; Pritchard et al., 1999). Even in the trimeric complex, the interaction between importin β and Rae1-Nup98 remained RanGTP-sensitive, suggesting that Nup98 serves as a bridging factor to mediate the regulation of the Rae1 complex by Ran and importin β.

Rae1 Is Present in an RNA-Dependent Complex that Regulates Microtubule Dynamics

Rae1 contains several highly conserved WD40 repeats (Pritchard et al., 1999) and is predicted to form a β propeller structure. It was initially identified as a factor required for mRNA export in yeasts *S. pombe* and *S. cerevisiae* (Brown et al., 1995; Murphy et al., 1996) and as a protein which can be crosslinked to poly(A) mRNA in cultured human cells (Kraemer and Blobel, 1997). Rae1 has also been shown to interact directly with Nup98 and TAP/Mex67, both of which are required for efficient mRNA export in many different systems (Blevins et al., 2003; Pritchard et al., 1999; Yoon et al., 2000). Recent studies have offered conflicting views of the role of Rae1 in mRNA export (Babu et al., 2003; Pritchard et al., 1999; Sitterlin, 2004) and have suggested that Rae1 may function primarily as a cell cycle checkpoint regulator (Babu et al., 2003) rather than an mRNA export factor. Interestingly, Rae1 was also shown to have an mRNA export-independent function in regulating mitotic progression in *S. pombe* (Whalen et al., 1997). While we did not address its potential role in mRNA export during interphase or its role as a mitotic checkpoint regulator, our data demonstrate that Rae1 is a component of an RNP required for spindle assembly in mitosis. It will be interesting to determine if the spindle-assembly defects we observe after Rae1 depletion are related to its potential role as a spindle assembly checkpoint regulator or if separate Rae1 complexes are responsible for these different activities.

The depletion of Rae1 from *Xenopus* extracts severely impaired the assembly of Ran asters and sperm spindles. These mitotic phenotypes are a direct consequence of the loss of Rae1 since they could be partially rescued by the addition of recombinant Rae1. Although the detailed mechanism of how Rae1 functions in spindle assembly is unclear, this function of Rae1 in mitosis appears to be conserved since the depletion of Rae1 by RNAi in HeLa cells also led to severe spindle-assembly defects.

Although recombinant Rae1 binds directly to taxol-stabilized purified microtubules, it does not appear to affect their organization. Based on our results, we therefore propose that Rae1, like other β propeller WD-repeat proteins, functions as a scaffolding platform that tethers functionally important factors to the mitotic microtubule network. Intriguingly, at least one of these factors appears to be an RNA-containing complex, suggesting that Rae1 mediates interaction between RNA and the mitotic spindle.

A Direct Role for RNA in Mitotic Spindle Assembly

The intimate interaction between Rae1 and RNA, RNA binding proteins and spindle microtubules, suggests a functional link between mitotic spindle morphology and RNP particles. We found that RNA, particularly ribosomal RNA, was enriched and specifically localized to mitotic microtubules. This result is reminiscent of prior studies demonstrating the presence of ribosomes on sea urchin embryo microtubules and the enrichment of specific mRNAs on these ribosomes (Hamill et al., 1994). This result also fits well with the observation that Rae1 associates with Maskin, which has been shown to bind both mRNA and microtubules (Groisman et al., 2000). It has been hypothesized that the association of Maskin and CPEB with the mitotic apparatus could regulate the local translation of mitotic regulators on the mitotic apparatus, which would lead to a localized enrichment of these proteins around spindle microtubules. This hypothesis is supported by the observation that the human homolog of Maskin, TACC-3, also functions in spindle assembly in human tissue culture cells (Gergely et al., 2003). Our data suggest that proteins such as Rae1 and Maskin that are involved in mRNA export and/or translational regulation may also play a more direct role in the regulation of the mitotic spindle, independent of local translation.

The use of *Xenopus* CSF extracts allowed us to examine the translation-independent spindle functions of RNAs and proteins. These extracts contain a large maternal storage pool and do not require transcription or translation for correct spindle assembly. While translation is not essential for spindle assembly in extracts, it appears to be required for full histone H1 kinase activity. This suggests that cyclin B mRNA is actively being translated in *Xenopus* extracts and that perhaps other critical mitotic regulators are also translated continuously. It will be interesting to determine if there are other messages that are actively translated during mitosis, whether their translation is localized to the mitotic apparatus, and if this translation contributes to mitotic fidelity in other cell types that do not contain large maternal stores of RNA and protein.

Although RNA has essential functions in many cellular processes, our results revealing a translation-independent role of RNA in mitotic spindle assembly were surprising, since the mitotic spindle apparatus was previously considered to be a purely protein-based molecular machine. We demonstrate that a large number of RNAs associate with the mitotic spindle and provide evidence that RNA itself is required for spindle morphogenesis. Moreover, Rae1 appears to function in an RNP, and RNA is not only essential for the integrity but also for the microtubule nucleation/stabilization activity of a purified Rae1 complex. Intriguingly, Rae1 only partially colocalizes with spindle-associated RNAs, and the phenotype of RNase treatment of Rae1 beads is less severe than the phenotype of RNase treatment of total extracts. This suggests that RNA may play a role in the function of multiple microtubule-associated protein complexes and that RNPs may have a more general role in the regulation of spindle assembly and morphology.

Functional Interplay between RNA and Microtubules

The specific localization of RNAs is a widely utilized mechanism to spatially regulate gene expression. This is known to play an important role in developmental cell fate decisions and in most cases is dependent on either the actin or microtubule cytoskeleton (Tekotte and Davis, 2002). The specific association of RNAs with the mitotic spindle apparatus could have several possible explanations. First, interaction with the spindle could allow for the local translation of specific mitotic regulators, enriching these proteins at their site of action in the absence of transport, which in turn could be important for correct cell division. Secondly, specific mRNAs may be tethered to the spindle in order to guarantee their correct distribution in the case of asymmetric cell divisions. Alternatively, it could ensure the equal distribution of RNAs to dividing daughter cells, which may be important for critical but low-abundance transcripts. A fourth possibility could be that certain RNAs play a structural or functional role in the assembly process or the regulation of the spindle apparatus. Such an intriguing model is supported by our data that provide evidence for a mechanistic role for RNA in the process of spindle assembly. This concept is novel in that it highlights an active role for RNP particles in cell division. It has generally been assumed that RNPs are passive cargo along cytoskeletal polymers, but our work suggests that RNPs in turn can also influence cytoskeletal dynamics. How could RNA function in spindle assembly and contribute to mitotic fidelity? First, as seen for the Rae1-containing RNP, RNA may serve as an integral complex component, which may be necessary for complex stability or integrity. Second, it is possible that RNA plays a more active role. For example, the binding of RNA to microtubule-associated proteins may directly influence their activity or their rate of turnover. In the case of microtubule-based motor proteins, large RNP particles may significantly influence their velocity of movement or other aspects of their activities. Although the RNPs may not be in a filamentous network, they could crosslink or provide a scaffold for factors involved in spindle function. Future experiments are necessary to elucidate the active and passive roles of RNA in mitotic spindle assembly and function.

Experimental Procedures

In Vitro Protein Interaction Assays

Purified recombinant proteins were mixed at final concentrations of the following: 10 nM Rae1, 10 nM GST-Nup98, S-importin β 1–876 (0–320 nM); reactions containing Ran also contained 20 or 200 nM RanQ69L, 100 nM hRCC1, and 1 mM GTP. Reactions were per-

formed in a 200 μl volume of PBS + 10 mg/ml BSA. Proteins were mixed and incubated for 30 min at room temperature, followed by addition of 20 μl of S protein agarose (Novagen) and a further incubation for 30 min at room temperature. Pelleted beads were washed 5 × 1.5 ml × 3 min with PBS. Captured proteins were analyzed by Western blotting.

Immunodepletion of Rae1 from CSF Extracts

Anti-Rae1 (1926 or 2238) or nonspecific rabbit IgG (Sigma) antibodies were prebound to Protein A Dynabeads (Dynal) at a ratio of 25 μg Ab/100 μl of beads. Fifty microliters of CSF extract was depleted with 50 μg Ab beads for 1 hr on ice. Beads were collected using a magnet (Dynal), and the supernatant from the IP was used to assess Ran aster and sperm-spindle assembly. For rescue of depletions, rRae1 was added to extracts at a concentration of 100 nM. For rescue of the Rae1 depletion effect on Ran aster assembly, fractions from the final SP column (either fraction 7, which contained peak activity, or fraction 10, which contained no activity) were added to control and Rae1-depleted reactions at a dilution of 1:10. The significance of the effect of the addition of recombinant Rae1 to extracts was analyzed using a Student's t test in Microsoft Excel.

Anti-Rae1 Bead Microtubule Nucleation Assays

Five micrograms of anti-Rae1 (2238) or rabbit IgG were bound to 20 μl of Protein A Dynabeads. These antibodies were used to immunoprecipitate Rae1 from 10 μl of the 28% amonium sulfate pellet dissolved in EB^+ for 1 hr at 4°C. The beads were washed 3 × 1 ml with EB + 500 mM KCL, 1 × 1 ml with EB, and the equivalent of 1 μg of antibody-coated beads was added to 5 λl of double-depleted extract (ΔRanBPΔimportin β [71–601]). The extract and beads were incubated for 16 min at 20°C and spun onto coverslips. For inhibition of Rae1-coated beads by importin β, the washed beads were incubated with 20 μM importin β [71–876] or [71–601] for 30 min at room temperature. The beads were washed and incubated in double-depleted extract as described above. For inhibition of Rae1 activity with RnaseA, the ammonium sulfate pellet was incubated with 100 μg/ml RNaseA for 30 min at room temperature prior to immunoprecipitation. Each experiment was repeated four times to ensure reproducibility.

RNase Treatment of Extract and RNA Analysis

RNaseA (Sigma) was dissolved at 10 mg/ml in SDB (5 mM HEPES [pH 7.7], 100 mM KCl, 1 mM $MgCl_2$, 150 mM sucrose) and boiled for 10 min. Boiled RNaseA was centrifuged at 20,000 × g for 15 min and frozen in small aliquots. S protein (Sigma) was dissolved in SDB at 5 mg/ml; S peptide was dissolved in SDB. RNaseA (Sigma) was coupled to CNBr Activated Sepharose beads (Amersham Pharmacia) at a concentration of ~5 mg/ml of beads as described by the manufacturer.

Extracts were treated with RNase by adding RNase to extract at a concentration of 100 μg/ml, S protein to 100 μg/ml, S peptide to 20 μg/ml, or S peptide premixed with S protein to 100 μg/ml and incubating at room temperature (~21°C) for 1 hr. Extract was treated with RNase beads by incubating 100 μl of extract with 20 μl of RNase beads (or unmodified CNBr beads) for 1 hr at room temperature. RNA from total extract was isolated by proteinase K digestion, phenol/chloroform extraction, followed by ethanol precipitation. To isolate microtubule-associated RNAs, microtubules were induced to polymerize (by addition of RanQ69L, taxol, or sperm nuclei), and a 50 μl reaction was diluted with 1 ml of BRB80 + 30% glycerol. The diluted reaction was overlayed on a 4 ml cushion of BRB80 + 60% glycerol and centrifuged for 15 min at 17,000 × g. The sample cushion interface was washed 2 times with water and the pellet was resuspended in water. RNA was extracted as described for total extract. Ten micrograms of RNA was analyzed by agarose gel electrophoresis and ethidium bromide staining.

Supplemental Experimental Procedures are available with the Supplemental Data from this paper.

Supplemental Data

Supplemental Data include seven figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/121/2/223/DC1/.

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References

Babu, J.R., Jeganathan, K.B., Baker, D.J., Wu, X., Kang-Decker, N., and van Deursen, J.M. (2003). Rae1 is an essential mitotic checkpoint regulator that cooperates with Bub3 to prevent chromosome missegregation. J. Cell Biol. *160*, 341–353.

Blevins, M.B., Smith, A.M., Phillips, E.M., and Powers, M.A. (2003). Complex formation among the RNA export proteins Nup98, Rae1/Gle2, and TAP. J. Biol. Chem. *278*, 20979–20988.

Brown, J.A., Bharathi, A., Ghosh, A., Whalen, W., Fitzgerald, E., and Dhar, R. (1995). A mutation in the Schizosaccharomyces pombe rae1 gene causes defects in poly(A)+ RNA export and in the cytoskeleton. J. Biol. Chem. *270*, 7411–7419.

Budde, P.P., and Heald, R. (2003). Centrosomes and kinetochores, who needs 'Em? The role of noncentromeric chromatin in spindle assembly. Curr. Top. Dev. Biol. *56*, 85–113.

Deshler, J.O., Highett, M.I., and Schnapp, B.J. (1997). Localization of Xenopus Vg1 mRNA by Vera protein and the endoplasmic reticulum. Science *276*, 1128–1131.

Fontoura, B.M., Blobel, G., and Yaseen, N.R. (2000). The nucleoporin Nup98 is a site for GDP/GTP exchange on ran and termination of karyopherin beta 2-mediated nuclear import. J. Biol. Chem. *275*, 31289–31296.

Gergely, F., Draviam, V.M., and Raff, J.W. (2003). The ch-TOG/XMAP215 protein is essential for spindle pole organization in human somatic cells. Genes Dev. *17*, 336–341.

Groisman, I., Huang, Y.S., Mendez, R., Cao, Q., Theurkauf, W., and Richter, J.D. (2000). CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: Implications for local translational control of cell division. Cell *103*, 435–447.

Gruss, O.J., Carazo-Salas, R.E., Schatz, C.A., Guarguaglini, G., Kast, J., Wilm, M., Le Bot, N., Vernos, I., Karsenti, E., and Mattaj, I.W. (2001). Ran induces spindle assembly by reversing the inhibitory effect of importin alpha on TPX2 activity. Cell *104*, 83–93.

Hamill, D., Davis, J., Drawbridge, J., and Suprenant, K.A. (1994). Polyribosome targeting to microtubules: enrichment of specific mRNAs in a reconstituted microtubule preparation from sea urchin embryos. J. Cell Biol. *127*, 973–984.

Harel, A., and Forbes, D.J. (2004). Importin beta: Conducting a much larger cellular symphony. Mol. Cell *16*, 319–330.

Heald, R., Tournebize, R., Blank, T., Sandaltzopoulos, R., Becker, P., Hyman, A., and Karsenti, E. (1996). Self-organization of microtubules into bipolar spindles around artificial chromosomes in Xenopus egg extracts. Nature *382*, 420–425.

Kahana, J.A., and Cleveland, D.W. (1999). Beyond nuclear transport. Ran-GTP as a determinant of spindle assembly. J. Cell Biol. *146*, 1205–1210.

Karsenti, E., and Vernos, I. (2001). The mitotic spindle: a self-made machine. Science *294*, 543–547.

Kirschner, M., and Mitchison, T. (1986). Beyond self-assembly: From microtubules to morphogenesis. Cell *45*, 329–342.

Kraemer, D., and Blobel, G. (1997). mRNA binding protein mrnp 41 localizes to both nucleus and cytoplasm. Proc. Natl. Acad. Sci. USA *94*, 9119–9124.

Kroll, T.T., Zhao, W.M., Jiang, C., and Huber, P.W. (2002). A homolog of FBP2/KSRP binds to localized mRNAs in Xenopus oocytes. Development *129*, 5609–5619.

Lieb, B., Carl, M., Hock, R., Gebauer, D., and Scheer, U. (1998). Identification of a novel mRNA-associated protein in oocytes of Pleurodeles waltl and Xenopus laevis. Exp. Cell Res. *245*, 272–281.

Murphy, R., Watkins, J.L., and Wente, S.R. (1996). GLE2, a Saccharomyces cerevisiae homologue of the Schizosaccharomyces pombe export factor RAE1, is required for nuclear pore complex structure and function. Mol. Biol. Cell *7*, 1921–1937.

Nachury, M.V., Maresca, T.J., Salmon, W.C., Waterman-Storer, C.M., Heald, R., and Weis, K. (2001). Importin beta is a mitotic target of the small GTPase Ran in spindle assembly. Cell *104*, 95–106.

Pritchard, C.E., Fornerod, M., Kasper, L.H., and van Deursen, J.M. (1999). RAE1 is a shuttling mRNA export factor that binds to a GLEBS-like NUP98 motif at the nuclear pore complex through multiple domains. J. Cell Biol. *145*, 237–254.

Sampath, S.C., Ohi, R., Leismann, O., Salic, A., Pozniakovski, A., and Funabiki, H. (2004). The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly. Cell *118*, 187–202.

Sitterlin, D. (2004). Characterization of the Drosophila Rae1 protein as a G1 phase regulator of the cell cycle. Gene *326*, 107–116.

Tekotte, H., and Davis, I. (2002). Intracellular mRNA localization: motors move messages. Trends Genet. *18*, 636–642.

Whalen, W.A., Bharathi, A., Danielewicz, D., and Dhar, R. (1997). Advancement through mitosis requires rae1 gene function in fission yeast. Yeast *13*, 1167–1179.

Wiese, C., Wilde, A., Moore, M.S., Adam, S.A., Merdes, A., and Zheng, Y. (2001). Role of importin-beta in coupling Ran to downstream targets in microtubule assembly. Science *291*, 653–656.

Yoon, J.H., Love, D.C., Guhathakurta, A., Hanover, J.A., and Dhar, R. (2000). Mex67p of Schizosaccharomyces pombe interacts with Rae1p in mediating mRNA export. Mol. Cell. Biol. *20*, 8767–8782.

Supplemental Data

A Rae1-Containing Ribonucleoprotein Complex

Is Required for Mitotic Spindle Assembly

Michael D. Blower, Maxence Nachury, Rebecca Heald, and Karsten Weis

Supplemental Experimental Procedures

Double Depletion of Extracts and Purification of APA-β

Xenopous egg extracts were depleted with RanQ69L and importin β [71-601] columns as described (Nachury et al., 2001). A Schematic for the purification of the APA-β activity is displayed in Supplemental Figure S1, and details of the purification procedure are available upon request. The polypeptides pshown in Fig. 1 were identified by mass spectrometry in the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford. For the use of peak APA-β fractions in the rescue of Rae1 depleted extracts final column fractions were supplemented with 2mg/ml BSA and 5% glycerol and stored at -80°.

Cloning and Protein Expression

The following constructs were expressed in *E. coli* as 6His or GST fusion proteins and purified as described: S-tagged Importin β 1-876 (pKW485), S-tagged Importin β 71-876 (pKW488), S-tagged importin β 71-601 (pKW762), S-tagged importin β 71-405 (pKW1290), Ran Q69L (pKW590), hNup98 (Fontoura et al., 2000), xRae1(pKW1388).

To generate soluble recombinant Rae1 (rRae1), Rae1 was PCR amplified from pKW1388 (using primers UC1066 and 1067) and cloned into the NcoI and XhoI sites of pFBHTA (Invitrogen) (resulting plasmid is pKW1389). Bacemid and virus were produced according to manufacturer's instructions. rRae1 was purified from bacculovirus infected Sf9 cells using NiNTA chromatography.

Antibody Generation and Purification

Two sets of antibodies were generated against xRae1. Antibodies 1925 and 1926 were generated by immunizing rabbits (Covance) with denatured Rae1 expressed in bacteria. Antibodies 2237 and 2238 were generated by immunizing rabbits (Covance) with soluble Rae1 expressed from Sf9 cells. Both sets of antibodies were affinity purified against rRae1 (from bacculovirus infected Sf9 cells) immobilized on an Affiprep column support (BioRad). All affinity purified Rae1 antibodies were used at 1μg/ml for immunofluorescence and 0.1 μg/ml for western blot. All antibodies gave similar results for Western and IF.

Pull-Downs from Extract

Recombinant S-tagged importin β proteins (10 μM) were added to CSF extracts (50 μL) and incubated for 30 minutes on ice (either + or – 25 μM RanQ69L), extracts were added to 50 μL S-protein agarose and incubated for 30 minutes on ice. Pelleted beads were washed 5 X 1.5 ml X 3 minutes with XB200 at 4°. Captured proteins were analyzed by western blotting.

Extract Preparation and Utilization

CSF arrested extracts were made essentially as described (Desai et al., 1999; Murray, 1991). Rhodamine or FITC labeled tubulin (Hyman, 1991) was prepared from pig brain was added to a final concentration of 0.2 mg/ml. CSF spindles and cycled spindles were generated as described (Desai et al., 1999), and Ran asters were generated by the addition of Ran Q69L to extracts at a concentration of 25 μM.

For analysis of Ran asters and spindles assembled in experimentally manipulated extracts reactions (20 μL) were centrifuged onto coverslips as described (Desai et al., 1999; Murray, 1991). For each experiment at

least 10 fields (Ran asters) (40X objective) or 100 sperm nuclei were quantitated. All extract experiments were repeated with multiple different extracts to ensure reproducibility.

HeLa Cell siRNA

HeLa cells were culture in DMEM (Invitrogen) according to standard procedures. HeLa cells were transfected with Rae1 siRNA oligos (Dharmacon) using Oligofectamine (Invitrogen) as described (Elbashir et al., 2002). The siRNA sequences used were: GCAGUAACCAAGCGAUACA (Rae1_05) and GAGUUGCUAUUCACUAUAU (Rae1_07). Both siRNA oligos silenced Rae1 expression and resulted in similar phenotypes, but Rae1_05 gave a more efficient knockdown. All results presented in Figure 5 come from cells treated with Rae1_05. As a control siRNA oligos directed against *Xenopus* Rae1, which were not present in the human Rae1 sequence, were used. Transfected cells were fixed and analyzed by Western blot and immunofluorescence for tubulin, Pericentrin (Covance), Rae1, and PH3 at 72 hours. For Pericentrin staining cells were fixed in –20 methanol for 10', for Tubulin and Rae1 staining cells were prepermebalized with 1% TritonX-100 in BrB80 for 2 minutes at 37°, then fixed with 4% paraformaldehyde, 0.1% glutaraldehyde in 1X BrB80 for 7 minutes at 37°. Mitotic index was scored as the number of PH3 positive cells out of 1000 total cells counted.

In Vitro Tubulin Binding

In vitro binding of rRae1 to taxol stabilized microtubules was performed essentially as described (Cheeseman et al., 2001). Recombinant proteins were added at the following concentrations: 10 nM Rae1, 10 nM GST-Nup98, and 5 μM importin β 1-876. Equivalent volumes of supernatant and pellet were analyzed by SDS-PAGE and Western blotting.

For visual immunofluorescence analysis of Rae1 binding to microtubules binding reactions were performed as described above, but the reaction was overlayed onto a 4 ml cushion of 40% glycerol made in 1X BrB80. Reactions were spun onto coverslips as described for sperm spindles and Ran asters. The binding of Rae1 to microtubules was analyzed by immunofluorescence.

H1 Kinase Assays

H1 kinase activity from treated extracts was analyzed using a filter binding assay as described (Felix et al., 1989). For some experiments cyclin BΔ90 was added to the extract at a concentration of 1μM at the start of the experiment.

Cytology, Microscopy, and Image Analysis

Immunofluorescence was performed essentially as described (Blower and Karpen, 2001).

Immunofluorescence analysis of taxol stabilized microtubules was performed by centrifuging microtubules onto coverslips as described for sperm spindles (Desai et al., 1999), and staining for Rae1 and α-tubulin (E7 monoclonal).

RNA was stained on sperm spindles and Ran asters by mounting fixed samples in Vectashield (Vector Labs) containing 1 μg/ml Propidium Iodide and 1 μg/ml DAPI.

Images were aquired using a Nikon eclipse E600 microscope equipped a Hamamatsu digital camera CA742-98 using the Metamorph Software program (Universal Imaging). Images in Figure 5 were aquired using a Deltavision Spectris imaging system.

Supplemental References

Blower, M.D., and Karpen, G.H. (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. Nat. Cell. Biol. *3*, 730–739.

Desai, A., Murray, A., Mitchison, T.J., and Walczak, C.E. (1999). The use of Xenopus egg extracts to study mitotic spindle assembly and function in vitro. Methods Cell Biol. *61*, 385–412.

Elbashir, S.M., Harborth, J., Weber, K., and Tuschl, T. (2002). Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods *26*, 199–213.

Felix, M.A., Pines, J., Hunt, T., and Karsenti, E. (1989). A post-ribosomal supernatant from activated Xenopus eggs that displays post-translationally regulated oscillation of its cdc2+ mitotic kinase activity. EMBO J. *8*, 3059–3069.

Hyman, A.A. (1991). Preparation of marked microtubules for the assay of the polarity of microtubule-based motors by fluorescence. J. Cell Sci. *Suppl. 14*, 125–127.

Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.

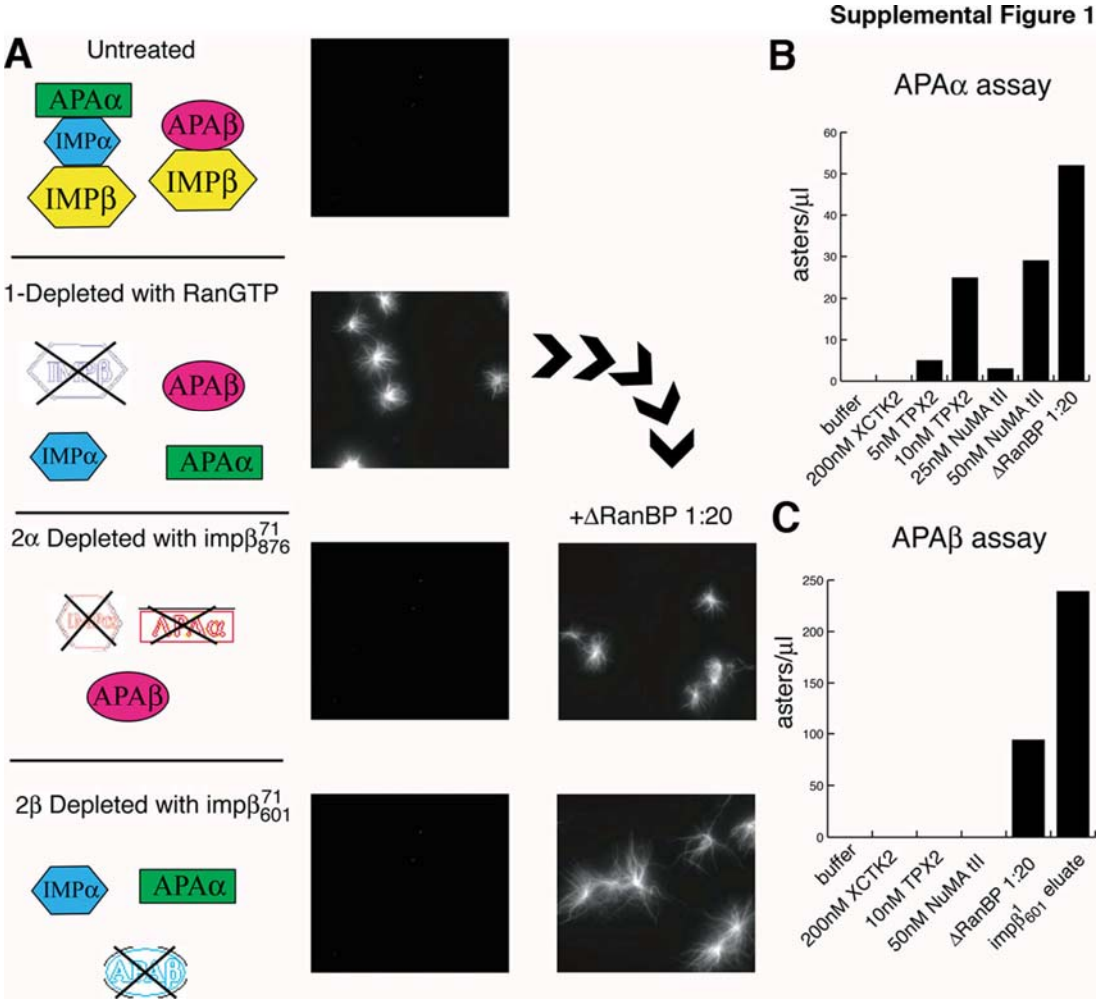


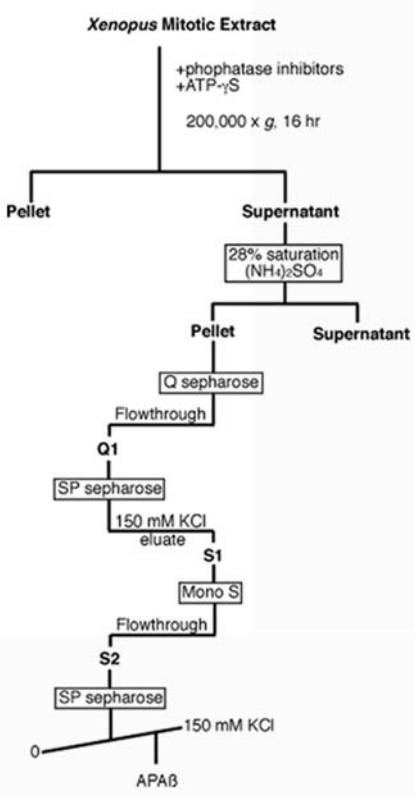
Figure S1. Assays and Purification Scheme for APA-β, the Importin α-Independent Aster-Promoting Activity

(A) Scheme illustrating the assay used to identify both mitotic cargoes regulated by both importin α/β (APA-α, for Aster Promoting Activity regulated by importin α) or directly by importin β (APA-β, for Aster Promoting Activity regulated by importin β). Extracts were depleted with RanQ69L, and formed spontaneous microtubule asters due to the liberation of both classes of importin β regulated cargoes (APA-α and APA-β). 2 α. Extracts were then depleted with importin β [71-876], which depleted APA-α from the extract. Of note, although APA-β should also be depleted by this procedure, we found that Rae1 was depleted much less efficiently with importin β 71-876 than with importin β 71-601. 2 β. Depletion with

importin β [71-601] removed APA-β but not APA-α. Aster formation in these depleted extracts could be rescued by the addition of either the ΔRanBP extract or diluted high-speed extract.
(B) The aster forming activity of the ΔRanBPΔimportin β [71-876] double depleted extract was monitored after the addition of the indicated concentrations of recombinant protein or diluted ΔRanBP extract.
(C) The aster forming activity of the ΔRanBPΔimportin β [71-601] double depleted extract was monitored after the addition of either the indicated concentrations of recombinant protein, diluted ΔRanBP extract or an eluate of an importin β [1-601] column. This result demonstrates that APA-α and APA-β are distinct and there is a NuMA- and TPX2-independent activity that is removed from the extract by importin β [71-601].

Supplemental Figure 2

A



B

Fraction	Enrichment	Yield
Mitotic Extract	1	100%
$(NH_4)_2SO_4$ Pellet	8.2	80%
Q Sepharose FT	28.7	40%
SP Sepharose 150mM KCl eluate	143.5	32%
Mono S FT SP Sepharose eluate	~2870	22%

Figure S2. Purification Scheme for APA-β, the Importin α-Independent Aster-Promoting Activity
(A) Biochemical purification scheme used to purify APA-β from *Xenopus* CSF extract.
(B) Table indicating the enrichment and yield of APA-β at each step of the purification procedure. We estimated that the concentration of Rae1 in the peak APA-β fractions was ~500 pM. Recombinant Rae1 was unable to rescue the double depleted extract when added at 100 nM (the endogenous concentration), while the peak APA-β fractions could rescue the double depleted extract with ~12.5 pM Rae1. 50 pM Rae1 purified from extracts using the APA-β purification protocol was also able to completely rescue the Rae1 depleted extract, while 100 nM recombinant Rae1 gave only a partial rescue.

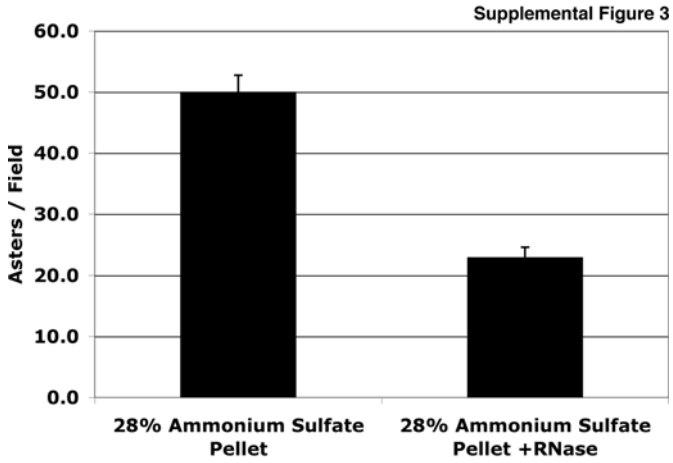


Figure S3. RNase Treatment Reduces the APA Activity of the Ammonium Sulfate Fraction in the APA-β Assay

The dissolved ammonium sulfate fraction (Figure S2) was treated with RNase as described in the Experimental Procedures and assayed for APA-β activity in the double-depleted extract (Figure 1) and the number of asters/field were scored. n = 5 experiments.

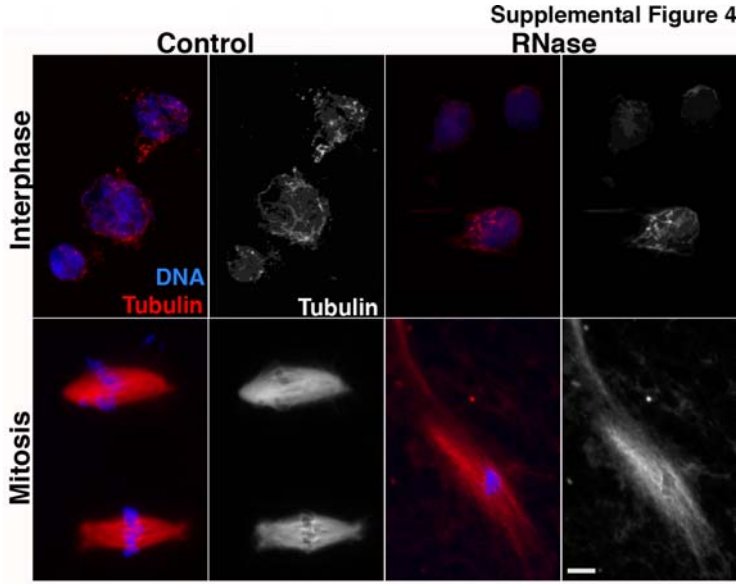


Figure S4. RNase Treatment Does Not Affect the Formation of Interphase Nuclei

Control or RNase treated extracts were released into interphase by the addition of Ca^{++}. Interphase chromatin decondensed normally and nuclei were similar to controls except that RNase treated nuclei were consistently smaller. When interphase extracts were cycled into mitosis by the addition of fresh RNase treated CSF arrested extracts, severe spindle assembly defects were observed in RNase treated samples. Microtubules are labeled in red, DNA is blue. Scale bar is 10 μm.

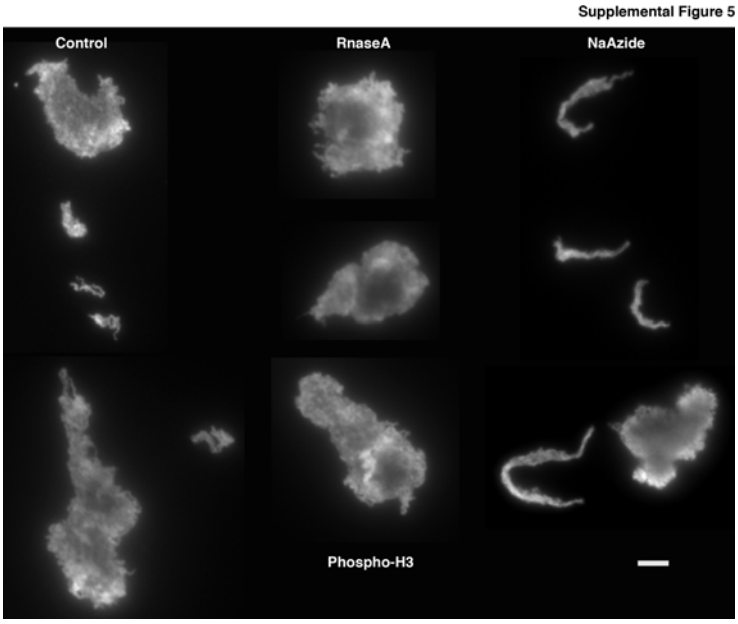


Figure S5. RNase Treatment Does Not Affect Chromosome Condensation
Control**,** RNase, and NaAzide treated CSF extracts were supplemented with sperm nuclei and chromosome condensation was monitored by phospho-histone H3 (PH3) immunofluorescence. Control and RNase treated extracts showed indistinguishable chromatin morphology, while NaAzide treated extracts showed little or no sperm decondensation. In these images nuclei were cropped from several images and assembled into this figure in order to present multiple examples of nuclear morphology. Scale bar is 10 μm.

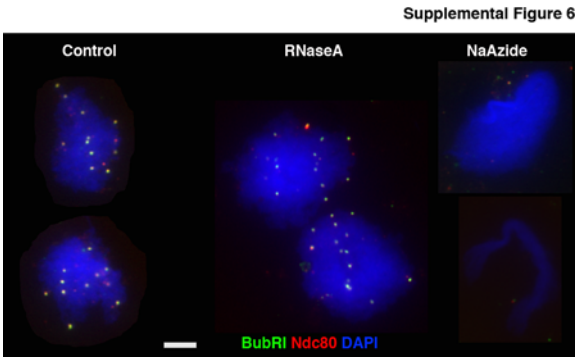


Figure S6. RNase Treatment Does Not Inhibit Kinetochore Assembly
Control, RNase, and NaAzide treated extracts were supplemented with sperm nuclei and kinetochore assembly was analyzed by staining for BubRI and Ndc80. Control and RNase treated nuclei showed prominent BubRI and Ndc80 staining on all nuclei, while NaAzide treated extracts showed essentially no kinetochore staining. In these images nuclei were cropped from several images and assembled into this figure in order to present multiple examples of kinetochore assembly. BubR1 is pseudocolored in green, Ndc80 is red, and DNA is blue. Scale bar is 10 μm.

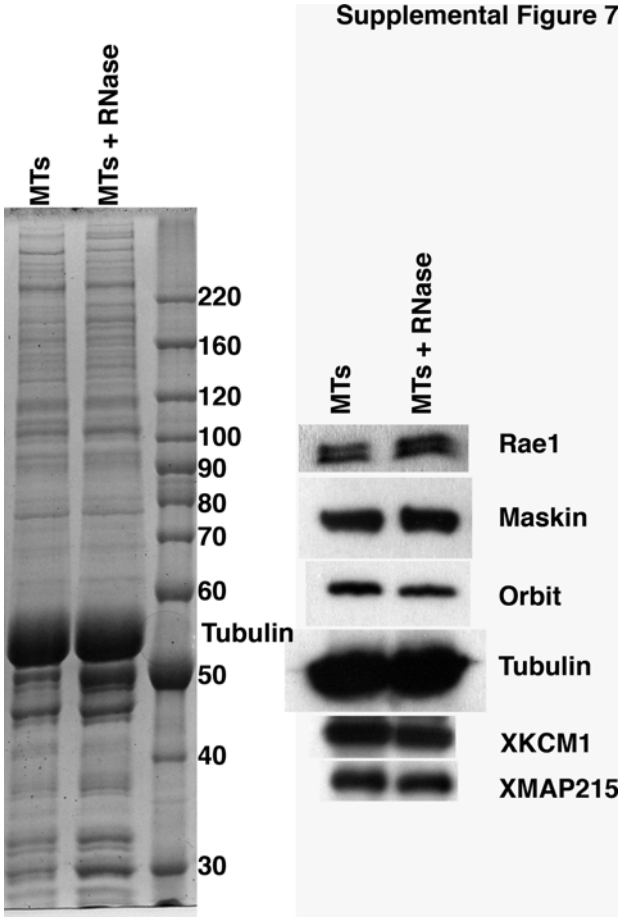


Figure S7. RNase Treatment Does Not Grossly Affect the Binding of MAPs to Taxol-Stabilized Microtubules

Microtubules were polymerized in crude CSF extract or CSF extract that was treated with RNase A by the addition of taxol (final concentration 10 μM). Microtubules were centrifuged through a 60% glycerol cushion as described in Experimental Procedures. Proteins from the MT pellet were run on a SDS-PAGE and stained with Coomassie blue. In parallel, fractions were also blotted for the microtubule-associated proteins indicated in the Figure. No gross differences were observed in the protein composition of microtubules isolated from extracts treated with RNase or control extracts.