dicating that the normal pathway for establishing silencing was not bypassed. Taken together, the results demonstrate that establishment of silencing involves something other than DNA replication during passage between early S and M phase.

A simple model to account for the findings is that activity of a critical silencing component, such as a Sir protein or silencer binding protein, is regulated in a cell cycle–dependent manner. Posttranslational modification of the protein(s), for example, might be required to initiate silent chromatin assembly. Necessary silencing activities might also be acquired by regulated synthesis of additional proteins or degradation of existing inhibitors. Although Sir proteins are not known to fluctuate during the cell cycle, their quantities are limiting, and redistribution of the factors from one location could influence silencing at another. Intriguingly, pools Sir3 and Sir4 were recently shown to partially disperse from their telomeric locations in mitotic cells (*25*).

A second model to account for the findings is that silencing requires chromatin remodeling or assembly factors that are not strictly associated with replication forks. According to this view, the factors might be targeted by silencers to specific locations where they would function sometime between early S and M phase. Alternatively, the presumed factors might promote global changes in chromatin structure during this period (e.g., mitotic condensation) that could also benefit silencing. Under certain conditions, such factors might also destabilize silent chromatin in a cell cycle–dependent manner. Indeed, transcriptionally repressed sequences from *HML* reactivated following excision from the chromosome in a replication-independent fashion during passage from G1 to M (*18*). Silencing of a telomere-proximal reporter gene was also shown to be least effective in cells arrested in M phase (*26*).

Recent work has linked CAF1 to heterochromatin in mammalian cells. The replication-coupled chromatin assembly factor associates with HP1 and accumulates in heterochromatic regions long after replication is complete (*27*–*29*). The factor also localizes to replication foci, where it is targeted by interaction with proliferating cell nuclear antigen (*30*). If the role for CAF1 in yeast silencing is distinct from its role at replication forks, a mechanism must exist to target the factor to silenced domains.

Last, the experiments performed here do not necessarily exclude a contributory role for replication in establishment of silent chromatin. Although silencing can occur in nonreplicating DNA, it is possible that passage of a replication fork accelerates the establishment process. This kinetic difference may be beneficial when silencing must occur within a rapid developmental program or in rapidly dividing cells.

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12. A LexA-based conditional silencer was obtained by replacing the Bam HI–Sph I fragment containing the ORC site in the synthetic *HMR* silencer of pJR1273 (*6*) with a PCR-amplified fragment containing 12 overlapping LexA sites from plasmid pAA6 (*31*). A Stu I–Xho I fragment containing the modified synthetic silencer and adjacent **a** genes was cloned between the *RS* sites in plasmid hmr::rHMR (*19*) to yield pLCY8. A similar procedure was used to create pLCY7, which contains six overlapping LexA sites. pLCY7 and pLCY8 were used to create strains YCL21 and YCL49, respectively, by regenerating the *HMR* locus in appropriately modified strains [see (*15*) for details].
13. A LexA-Sir1 expression vector (pMET3P-LexASIR1^{HIS3}) was obtained by replacing the Eco RV–Pst I fragment encoding the GAL4-binding domain and NH_2-terminus of *SIR1* (amino acids 1 to 136) in pJR1811 (*6*) with the LexA gene (Hind III–Xma I) from pBTM116 (*31*). The resulting chimera is expressed from the *MET3* promoter, which is induced by Met depletion. A vector expressing only LexA (pMET3P-LexA^{HIS3}) was obtained by deleting the remainder of the *SIR1* gene.
14. Cells were precultured overnight in SC-Leu-His containing 2% dextrose and 2 mM Met and then were inoculated into a similar medium containing raffinose (initial OD = 0.02). When the culture reached mid-log, galactose was added to a final concentration of 2% to induce recombinase expression. One hour later, cells were pelleted, washed, and resuspended in galactose-containing medium that lacked Met to induce expression of the LexA chimeras (*13*). Maximal excision (>80%) occurred within 1 hour, as noted previously (*19*).
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33. Whole-cell lysates were cleared with protein A agarose before antibody addition, according to the manufacturer's recommendation, to reduce nonspecific binding of chromatin. Antibodies for nonacetylated H3 (40 μl) and acetylated H4 (5 μl) were incubated with the precleared lysate for 6 hours. Twenty times as much lysate was used for immunoprecipitated samples as for input samples. Images of ethidium bromide–stained agarose gels were captured by charge-coupled device camera (UVP, Cambridge, UK) and quantified with IPlab Gel software (Signal Analytics, now Scanalytics, Fairfax, VA). Linear responses were obtained for the range of template concentrations used. Primer sequences are as follows: RING-1 (5′-GGCAAAGGCGATCCTCTAG-3′); RING-3b (5′-CAAACTTTGAGAGAAATATGTCTTTC-3′); ACT1-3 (5′-CTTCCACGTCCTCTTGCAT-3′); ACT1-3c (5′-GCGTGAAAAATCTAAAAGCTGATG-3′).

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Role of Importin-β in Coupling Ran to Downstream Targets in Microtubule Assembly

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The guanosine triphosphatase Ran stimulates assembly of microtubule asters and spindles in mitotic *Xenopus* egg extracts. A carboxyl-terminal region of the nuclear-mitotic apparatus protein (NuMA), a nuclear protein required for organizing mitotic spindle poles, mimics Ran's ability to induce asters. This NuMA fragment also specifically interacted with the nuclear transport factor, importin-β. We show that importin-β is an inhibitor of microtubule aster assembly in *Xenopus* egg extracts and that Ran regulates the interaction between importin-β and NuMA. Importin-β therefore links NuMA to regulation by Ran. This suggests that similar mechanisms regulate nuclear import during interphase and spindle assembly during mitosis.

In interphase of the cell cycle, the guanosine triphosphate (GTP)–bound form of the small guanosine triphosphatase, Ran, is concentrated in the nucleus, whereas its guanosine diphosphate (GDP)–bound form predominates in the cytoplasm (*1*). This gradient of

RanGTP is essential for the directional transport of proteins across the nuclear envelope, which involves assembly of specific import complexes in the cytoplasm. Importin-β bound to cargo directly, or indirectly through importin-α, constitutes one class of import complex. The cargo is released in the nucleus by binding of RanGTP, which induces a conformational change in importin-β and displaces the cargo (*1*–*4*).

In mitotic *Xenopus* egg extracts, RanGTP also stimulates microtubule aster and spindle formation (*5*–*8*). Because the nucleotide exchange factor for Ran, RCC1, is tethered to chromatin, a gradient of RanGTP might surround the mitotic chromosomes to serve as positional cue for spindle assembly. Consistent with this, RanGTP affects multiple microtubule regulators (*9*). Although the mechanism by which Ran regulates nuclear trafficking is well established, the mechanism by which RanGTP stimulates microtubule assembly in mitosis is unknown.

Spindle assembly is a highly regulated process that requires the coordination of plus- and minus-end–directed motor proteins (*10*) and nonmotor proteins such as NuMA (*11*, *12*) and TPX2 (*13*, *14*). NuMA organizes the minus ends of microtubules at spindle poles (*11*, *12*, *15*). It is a ~230-kD protein with a tripartite structure: a globular "head" domain, a coiled-coil "rod" domain, and a ~530–amino acid COOH-terminal globular "tail" domain that mediates oligomerization (*16*). NuMA "tail II" (the COOH-terminal 260 amino acids of *Xenopus* NuMA), like RanGTP, induces formation of microtubule asters when added to cytostatic factor (CSF)–arrested *Xenopus* egg extracts (*11*).

Microtubule structures induced by NuMA tail II closely resembled those induced by an activated form of Ran with a mutation in the effector domain, RanL43E (*5*, *17*) (Fig. 1A). No microtubule structures were assembled with other NuMA fragments (head, amino acids 1 to 251; rod, amino acids 627 to 1051; and tail I, amino acids 1719 to 1993 of *Xenopus* NuMA). Like RanL43E, NuMA tail II did not stimulate microtubule polymerization from pure tubulin (*18*), suggesting that NuMA tail II does not promote microtubule growth directly but instead requires additional factors.

To identify factors in CSF-arrested extracts involved in aster formation, we isolated proteins that interacted with NuMA tail II (Fig. 1B). Two proteins, about 55 and 95 kD in size, specifically bound to NuMA tail II. The ~95-kD protein migrated to a similar position in the gel, as did a protein that associated with RanL43E (Fig. 1B). To investigate whether the ~95-kD protein bound both RanL43E and NuMA tail II, we isolated proteins that associated with NuMA tail II (*19*) and probed them with GST-RanL43E. A ~95-kD protein specifically bound to RanL43E but not to controls (Fig. 1C). Its apparent molecular size and RanGTP-binding properties suggested that this protein was related to importin-β. This was confirmed by mass spectrometry (*20*) and by protein immunoblotting with polyclonal (*21*) (Fig. 1B) and monoclonal (Fig. 1C) antibodies specific for importin-β. The ~55-kD band was found by protein immunoblotting to be a mixture of tubulin and importin-α (*18*).

The association of importin-β with proteins that induce aster formation suggested a role for importin-β in microtubule assembly. Thus, we examined the effect of exogenously added importin-β on aster formation in the egg extract. Bacterially expressed, purified human importin-β (*22*) inhibited RanL43E-induced aster formation in a dose-dependent manner (Fig. 2, A and B). Importin-β also strongly inhibited the assembly of microtubule asters induced by NuMA tail II or by sperm centrosomes (Fig. 2A). The amount of importin-β required to cause complete inhibition of sperm asters ranged from 0.5 to 1.5 mg/ml, depending on the extract (six different extracts tested). The inhibitory effect appeared not to be due to general toxicity of importin-β, because taxol-induced microtubule formation was not inhibited (Fig. 2A). Addition of importin-α, which acts as an adaptor for importin-β in nuclear import, did not inhibit microtubule aster assembly (*18*).

If importin-β is an inhibitor of aster formation, its removal from the egg extract might result in spontaneous aster formation

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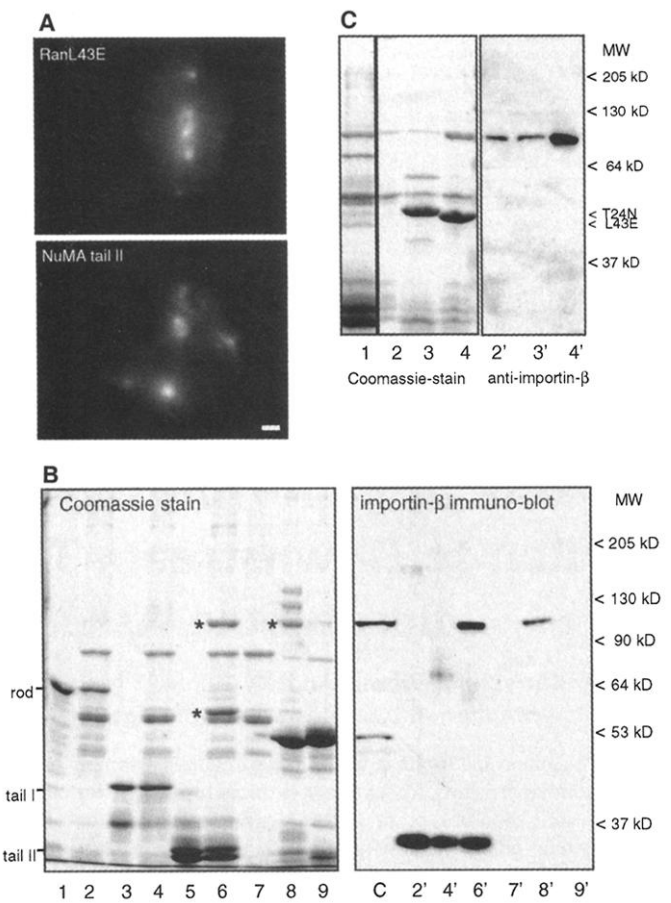

Fig. 1. NuMA tail II induces microtubule asters and binds to the Ran-binding protein importin-β in *Xenopus* egg extracts. (**A**) Microtubule structures formed in the presence of RanL43E or NuMA tail II (amino acids 1994 to 2253 of *Xenopus* NuMA) (*11*). Scale bar, 20 μm. (**B**) Specific interaction of NuMA tail II with two proteins in egg extracts. NuMA rod (lane 1), tail I (lane 3), tail II (lane 5), GST-RanL43E, or GST-RanT24N (a dominant negative allele of Ran resembling RanGDP) (*5*) were incubated with egg extracts. Proteins bound to NuMA rod (lanes 2 and 2′), tail I (lanes 4 and 4′), tail II (lane 6 and 6′), magnetic beads alone (lanes 7 and 7′), GST-RanL43E (lanes 8 and 8′), or GST-RanT24N (lanes 9 and 9′) were analyzed by Coomassie blue staining (left) or by protein immunoblotting with affinity-purified polyclonal antibodies to human importin-β (right) (*21*). These antibodies recognize importin-β and an unknown smaller protein of ~50 kD and cross-reacted nonspecifically with a ~30-kD bacterial protein (lanes 2′, 4′, and 6′). Lane C, extract control; asterisks, proteins that specifically bind to NuMA tail II (lane 6) or RanL43E (lane 8). (**C**) Binding of the ~95-kD protein to RanL34E but not RanT24N. NuMA tail II–binding proteins (lane 1) were incubated with glutathione beads alone (lanes 2 and 2′) or with GST-RanT24N (lanes 3 and 3′) or GST-RanL43E (lanes 4 and 4′) tethered to glutathione beads. Bound proteins were analyzed by Coomassie blue staining or by protein immunoblotting with monoclonal antibody to importin-β (Transduction Laboratories).

independent of RanL43E or NuMA tail II. We therefore depleted extracts of importin-β (along with other RanGTP-binding proteins) by incubation with GST-RanL43E (*23*). Depletion with RanL43E reduced the amounts of importin-β to 20 to 35% of those in control extracts incubated with wild-type RanGDP. Whereas aster formation depended on RanL43E in the control depleted extracts, microtubule asters formed spontaneously in the depleted extracts (Fig. 3A). Moreover, addition of RanL43E to the depleted extract had no effect on either the number or the intensity of the microtubule asters (Fig. 3, B and C), suggesting that the aster-forming activity was independent of RanGTP. Spontaneous aster formation was inhibited by the addition to the depleted extracts of either purified importin-β or a mutant importin-β lacking the NH_2-terminal 157 amino acids (ΔN–importin-β), which does not bind RanGTP (*22*, *24*, *25*) (Fig. 3). Thus, importin-β is a negative regulator of microtubule aster formation that acts downstream of RanGTP.

The observation that both NuMA tail II and Ran bind to importin-β suggests that

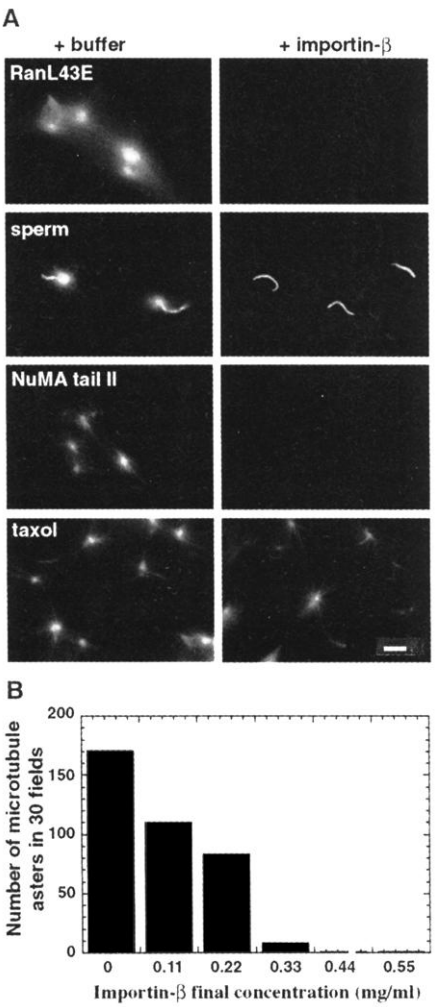

Fig. 2. Inhibition of microtubule aster formation by importin-β. (**A**) Microtubule structures formed in the absence (+ buffer) or presence of importin-β (0.55 mg/ml final) and GST-RanL43E (2 mg/ml), sperm chromatin, NuMA tail II (0.2 mg/ml), or taxol (10 μM) (*31*). Scale bar, 20 μm. (**B**) Quantification of the number of Ran-stimulated microtubule asters with increasing importin-β concentrations.

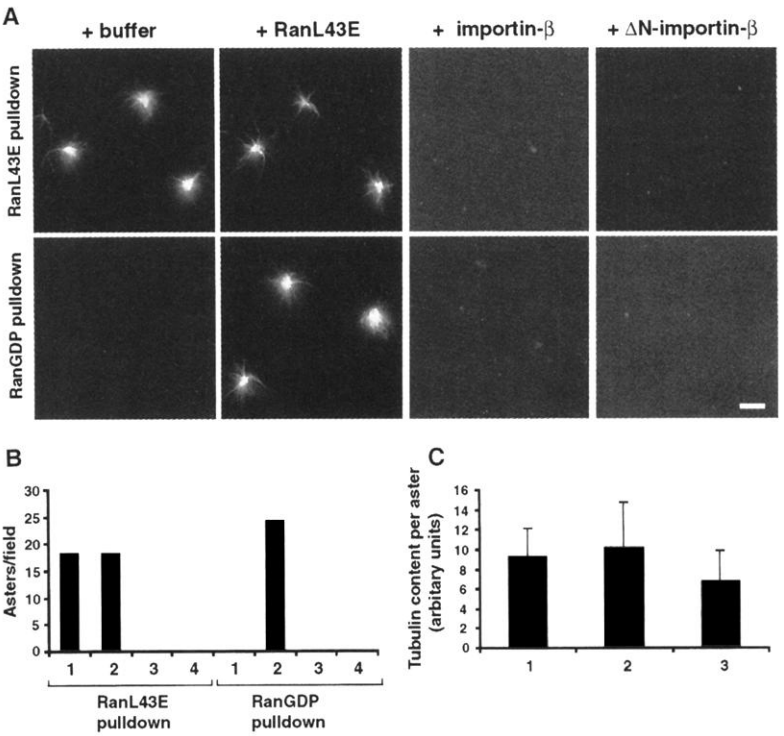

Fig. 3. Spontaneous microtubule aster formation in *Xenopus* egg extracts after removal of RanL43E-binding proteins. (**A**) Microtubule structures formed after depletion of proteins that bind to RanL43E or wild-type RanGDP. Samples were incubated with control buffer, RanL43E (1 mg/ml), importin-β (0.55 mg/ml), or ΔN–importin-β (1 mg/ml). Scale bar, 10 μm. (**B**) Average number of asters per field of view for depleted extracts incubated with 1, control buffer; 2, RanL43E; 3, importin-β; or 4, ΔN–importin-β. At least 30 fields were scored per sample. (**C**) Average tubulin content of each aster. 1, RanL43E pull down plus control buffer; 2, RanL43E pull down plus RanL43E; and 3, wild-type RanGDP pull down plus RanL43E. Error bars, standard deviation.

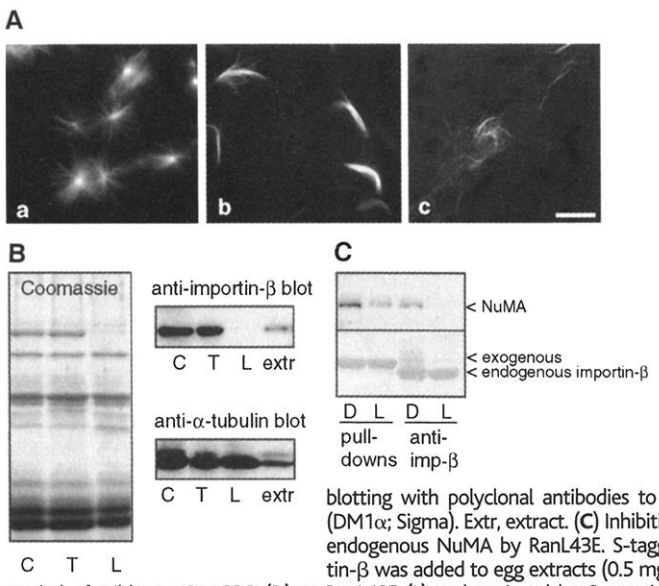

Fig. 4. Functional interaction of NuMA, importin-β, and Ran. (**A**) Ran-induced microtubule structures in extracts immunoprecipitated with control immunoglobulin (a) or antibodies to NuMA tail (b) or rod (c) (*11*, *12*). Scale bar, 50 μm. (**B**) Inhibition of importin-β binding to NuMA tail II in extracts treated with RanL43E. Tail II–binding proteins were isolated from egg extract incubated with buffer (C), GST-RanT24N (T), or GST-RanL43E (L) and analyzed by Coomassie blue staining or protein immunoblotting with polyclonal antibodies to importin-β (*21*) or α-tubulin (DM1α; Sigma). Extr, extract. (**C**) Inhibition of binding of importin-β to endogenous NuMA by RanL43E. S-tagged (Novagen) human importin-β was added to egg extracts (0.5 mg/ml final) in the presence of 1 mg/ml of wild-type RanGDP (D) or RanL43E (L) and retrieved by S-protein agarose beads (pull downs). Alternatively, proteins from extracts incubated with wild-type RanGDP (D) or RanL43E (L) were immunoprecipitated with monoclonal antibody to importin-β (anti-imp-β; Affinity Bioreagents). Bound proteins were analyzed by Western blotting with antibody to N-MA (*11*) and antibody to importin-β.

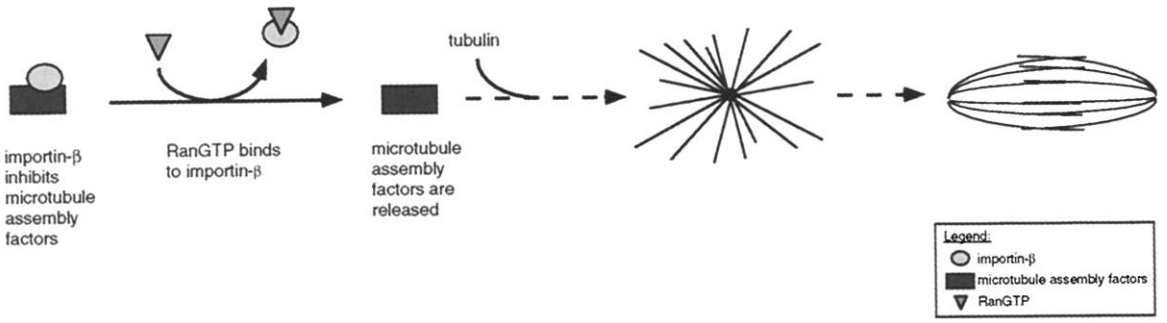

Fig. 5. Model for the mechanism of RanGTP-mediated microtubule aster formation through importin-β. RanGTP induces cargo release from importin-β, thus liberating proteins involved in microtubule aster and spindle formation.

NuMA may be in the same pathway as RanGTP. To test the relationship between NuMA and RanGTP in microtubule aster formation, we examined the effects of reducing the amount of endogenous NuMA in egg extracts. Consistent with a previous report that overexpression of full-length NuMA can overcome a defect in RCC1 function (*26*) (that is, a defect in production of RanGTP), a 30 to 40% reduction in the amount of endogenous NuMA (*27*) resulted in formation of microtubule "feathers" or disorganized microtubules (Fig. 4A). This suggests that NuMA activity is involved in organizing the minus ends of RanL43E-induced microtubules. NuMA therefore appears to function downstream of RanGTP in the aster assembly pathway.

The interactions of NuMA tail II and RanGTP with importin-β also suggested that importin-β might link Ran signaling to the function of NuMA in mitosis. We therefore examined the effects of RanL43E on the ability of NuMA tail II to bind to importin-β. Importin-β no longer bound to NuMA tail II when RanL43E was present in the extract (Fig. 4B). Therefore, Ran appears to disrupt the interaction between importin-β and NuMA tail II.

Immunoprecipitation of endogenous importin-β and pull-down experiments with recombinant importin-β (*28*) showed that NuMA associated with importin-β (Fig. 4C). Similarly, importin-β was associated with endogenous NuMA isolated by immunoprecipitation (*18*). This interaction was inhibited when RanL43E was added to the egg extract (Fig. 4C). This suggests that importin-β couples the effects of Ran to the microtubule-binding protein, NuMA, in egg extracts.

Considering the complexity of spindle assembly and considering that NuMA is partially dispensable for aster formation from sperm centrosomes (*11*), other mitotic microtubule regulators are likely to be regulated by Ran through importin-β. For example, the spindle pole–organizing protein, TPX2 (*13*, *14*), the microtubule-stabilizing protein, XMAP310 (*29*), and certain kinesins [such as XCTK2 (*30*)] also localize to the interphase nucleus and therefore might interact with importin-β. Indeed, addition of excess TPX2 to *Xenopus* egg extracts also induces microtubule aster formation (*14*). Our findings suggest that microtubule assembly proteins are inhibited by importin-β binding. Analogous to its role in the interphase nucleus, RanGTP could then stimulate aster formation by releasing the microtubule regulators from importin-β (Fig. 5).

The interaction between a nuclear import factor and mitotic spindle components may serve several functions. First, in interphase, nuclear import may sequester the spindle regulators and thus prevent disruption of the interphase microtubule organization by ectopic microtubule assembly. Second, nuclear accumulation of spindle regulators assures that they are present at high concentrations in the vicinity of the condensed chromosomes for spindle assembly at nuclear envelope breakdown. Third, we propose that during mitosis, importin-β directly inhibits the activities of microtubule regulators, therefore creating a generally suppressive environment for spindle assembly that is relieved only near the condensed chromosomes where there is a high local concentration of RanGTP. In organisms such as fungi, in which the nuclear envelope does not disassemble during mitosis, proteins involved in spindle formation must be imported into the nucleus during mitosis, suggesting a possible evolutionary origin of the relationship between nuclear protein import and spindle assembly.

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