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# Ran Is Required before Metaphase for Spindle Assembly and Chromosome Alignment and after Metaphase for Chromosome Segregation and Spindle Midbody Organization[D] [V]

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**The Ran pathway has been shown to have a role in spindle assembly. However, the extent of the role of the Ran pathway in mitosis in vivo is unclear. We report that perturbation of the Ran pathway disrupted multiple steps of mitosis in syncytial *Drosophila* embryos and uncovered new mitotic processes that are regulated by Ran. During the onset of mitosis, the Ran pathway is required for the production, organization, and targeting of centrosomally nucleated microtubules to chromosomes. However, the role of Ran is not restricted to microtubule organization, because Ran is also required for the alignment of chromosomes at the metaphase plate. In addition, the Ran pathway is required for postmetaphase events, including chromosome segregation and the assembly of the microtubule midbody. The Ran pathway mediates these mitotic events, in part, by facilitating the correct targeting of the kinase Aurora A and the kinesins KLP61F and KLP3A to spindles.**

## INTRODUCTION

During the cell cycle, the GTPase Ran regulates multiple cellular functions, including nucleocytoplasmic transport, nuclear envelope formation, and spindle assembly (Hetzer *et al*., 2002). The nucleotide-bound state of Ran (GTP or GDP) is spatially regulated by RCC1, the chromatin-bound guanine nucleotide exchange factor for Ran, and by RanGAP, which localizes to the cytoplasm and stimulates the intrinsic GTPase activity of Ran. The localization of these proteins is essential in regulating the activity of Ran throughout the cell cycle.

In vitro, RanGTP induces spindle assembly in mitotic *Xenopus* egg extracts in the absence of centrosomes, kinetochores, and chromatin (Carazo-Salas *et al*., 1999; Kalab *et al*., 1999; Ohba *et al*., 1999; Wilde and Zheng, 1999) by altering microtubule (MT) dynamics (Carazo-Salas *et al*., 2001; Wilde *et al*., 2001), changing the balance of motor activity (Wilde *et al*., 2001), and increasing centrosomal MT nucleation (Carazo-Salas *et al*., 2001). The Ran pathway can regulate spindle assembly by RanGTP binding to nuclear transport receptors (NTRs) and preventing them from interacting with and inhibiting spindle assembly factors (SAFs) that posses a nuclear localization signal (NLS) (Trieselmann *et al*., 2003; Tsai *et al*., 2003; Ems-McClung *et al*., 2004). For this process to drive spindle assembly, RanGTP must be generated at chromosomes where it maintains SAFs in an active form. Indeed, many in vivo and in vitro studies infer that RanGTP exists around mitotic chromatin and spindles, whereas NTRs have a much broader distribution resulting in the creation of an environment around chromosomes where NLS-containing SAFs are active (Kalab *et al*., 2002; Trieselmann and Wilde, 2002; Li and Zheng, 2004; Caudron *et al*., 2005).

To define the mitotic role of the Ran pathway in vivo, previous studies injected anti-RanBP1 antibodies or NTRs into mammalian cells (Guarguaglini *et al*., 2000; Nachury *et al*., 2001). However, these studies did not define the dynamic nature of the observed defects and may have missed the complete series of events that led to the observed phenotype at the time of fixation. In an alternative approach, RNA interference (RNAi) was used in *Caenorhabditis elegans* to reduce expression levels of different Ran pathway proteins (Askjaer *et al*., 2002; Bamba *et al*., 2002). These studies defined a role for the Ran pathway in the production of spindle MTs. However, because all spindle MT assembly was inhibited, these studies were unable to define any other mitotic processes regulated by Ran. Furthermore, because spindle assembly was not examined until at least 16–48 h after RNAi application, it is possible that the observed defects could result from the disruption of other cellular processes. Indeed, Bamba *et al*. (2002) showed that RNAi of Ran pathway components did inhibit nuclear transport. In addition, embryos and pronuclei were smaller after RNAi treatment for Ran pathway components (Askjaer *et al*., 2002).

To overcome these limitations, we used a microinjection approach to disrupt the Ran pathway just before mitosis and then immediately analyze the consequences of the perturbation on mitotic cellular function by time-lapse microscopy. By manipulating the Ran pathway in multiple ways and by microinjecting different concentrations of perturbants, we were able to "turn down" the pathway rather than completely or severely block it. This approach revealed multiple mitotic phenotypes that were not observed when all spindle MT production was inhibited (Askjaer *et al*., 2002; Bamba *et*

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Abbreviations used: MT, microtubule; NLS, nuclear localization signal; NTR, nuclear transport receptor; SAF, spindle assembly factor.

*al*., 2002). By comparing the effects with known phenotypes from different mutants and RNAi screens, we have generated an integrative view of how Ran regulates mitosis in syncytial *Drosophila* embryos. Indeed, we find that the Ran pathway regulates both pre- and postmetaphase events.

## MATERIALS AND METHODS

### *Fly Stocks*

*Drosophila melanogaster* lines used were wild type $w^{118}$ and lines expressing a fusion protein of green fluorescent protein (GFP) and α-tubulin (Grieder *et al*., 2000) and GFP and histone (Clarkson and Saint, 1999).

### *Recombinant Protein Expression and Purification*

Recombinant alleles of Ran and Ran pathway components fused to glutathione *S*-transferase were expressed in BL21 *Escherichia coli* and purified as described previously (Wilde and Zheng, 1999; Wiese and Zheng, 2000). Injected proteins were tested for their ability to bind to known factors and to support in vitro nuclear transport as described previously (Trieselmann and Wilde, 2002).

### *Generation of Anti-Aurora A Antibodies*

The Aurora A cDNA was amplified by PCR from expressed sequence tag clone LD 16949 and cloned into the BamHI and EcoRI sites of pGEX6P2. The GST-Aurora A fusion protein was expressed and purified as described above. The protein was further purified before antibody production by anion exchange chromatography and injected into rats to generate an anti-Aurora A polyclonal antibody (Pocono Rabbit Farm & Laboratory, Canadensis, PA). The anti-Aurora A antibody was affinity purified against the immunogen as described in Zhang *et al*. (2000) and was analyzed according to standard Western blot procedures (Supplemental Figure 1).

### *Quantification of Ran Concentration in Drosophila Embryos*

Serial dilutions of GST-Ran and of *Drosophila* embryo extracts were analyzed by SDS-PAGE and immunoblotted with an anti-Ran antibody (Cell Signaling Technology, Beverly, MA). The band intensity was analyzed using the histogram feature of Adobe Photoshop (Adobe Systems, Mountain View, CA).

### *RCC1 Binding Assay*

The binding assay was performed essentially as described previously (Trieselmann *et al*., 2003). Briefly, 1 μg of GST-RanT24N, rhodamine-labeled GST-RanT24N, or rhodamine-labeled GST was incubated with 1 μg of 6 His-RCC1 in the presence of glutathione agarose beads (Sigma-Aldrich, St. Louis, MO) for 1 h at 4°C. The beads were collected by centrifugation, washed three times, and the pellets and supernatants were analyzed by immunoblotting with an anti-His tag antibody (Cell Signaling Technology).

### *Embryo Microinjection*

Embryos were collected 2 h after they were laid, dechorionated with 50% bleach, aligned on agar plates, and dried for ~10 min (depending on room humidity) before injection. Small groups of embryos (~10) were injected with buffer or recombinant proteins, and the embryos were quickly viewed to determine what point of the cell cycle they were in. Embryos just about to enter mitosis, as judged by nuclear envelope breakdown, where then analyzed by time-lapse spinning disk confocal microscopy such that imaging began between 2 and 4 min after injection. Reagents were injected at ~1% of the total embryo volume. The initial concentrations of the injected proteins were 10 mg/ml wild-type Ran; 30 mg/ml RanBP1; 20 mg/ml CAS; 10 mg/ml RCC1; 32, 16, 8, or 5 mg/ml RanT24N; 32 mg/ml rhodamine-labeled RanT24N; 14 mg/ml importin α, 28 mg/ml ΔN importin β; 14 mg/ml importin α and 17 mg/ml importin β; 14 mg/ml importin α and 28 mg/ml ΔN importin β and 20 mg/ml RanGAP. By comparing Ran concentrations in embryos extracts to known concentrations of recombinant Ran by Western blotting, we estimated Ran to constitute 0.06% of embryonic protein or ~2 ng per embryo (Supplemental Figure 1). Because injected material constitutes 1–2% of the embryo volume (15 nl; Foe and Alberts, 1983), recombinant alleles of Ran were injected maximally at a fourfold excess. Timinszky *et al*. (2002) estimated that importin β constitutes 0.44 ng per embryo.

### *Staining of Microinjected Embryos*

Microinjected embryos were prepared for staining as described by Lamka and Lipshitz (1999). The primary antibodies were DM1A anti-α-tubulin (Sigma-Aldrich), YOl1/34 anti-α-tubulin (Sertotec, Oxford, United Kingdom), mAb1A1 anti-skeletor (gift from K. Johansen, Iowa State University, Ames, IA), anti-phospho-histone H3 antibody (Upstate Cell Signaling Solutions, Lake Placid, NY), anti-KLP3A (gift from M. Goldberg, Cornell University, Ithaca, NY), anti-phospho-KLP61F (gift from J. Scholey, University of California, Davis, Davis, CA), and anti-Aurora A kinase. The secondary antibodies conjugated to Alexa488 or Alexa568 were obtained from Molecular Probes (Eugene, OR). DNA was visualized by incubating embryos with TOTO-3 iodide (Molecular Probes) at 1:10,000 dilution for 10 min. Each staining was repeated at least three times.

### *Confocal Microscopy and Time-Lapse Imaging*

Images were captured with a Nikon Eclipse TE2000-E inverted microscope (Nikon Mississauga, Ontario, Canada) equipped with an UltraVIEW confocal spinning disk (PerkinElmer Life Sciences and Analytical Sciences, Missisauga, Ontario, Canada) and a Hamamatsu Orca-ER camera (Hamamatsu Photonics, Hamamatsu, Japan) using a Plan Apochromat 40× 1.5/numerical aperture 1.0 oil immersion lens. Images were acquired between 2 and 4 min after injection, in the median part of the embryo, at room temperature, every 8 s at three to five Z series of 40-μm steps using the MetaMorph software (Universal Imaging, West Chester, PA).

### *Data Analysis*

About 50% of the spindles and 10% of separating chromosomes were measured at random by drawing equally spaced parallel lines to the anterior–posterior axis of the embryos and choosing spindles or separating chromosomes that were on the lines. Chromosome separation velocities were obtained by measuring the distance between the leading edge of the separating chromosome masses at 8-s intervals and by calculating the slope of the average plot. Velocities of chromosome movement to poles were obtained by dividing chromosome separation velocities by 2. Measurements were made with the Region measurement feature of MetaMorph, and statistical analysis was done with Microsoft Excel (Microsoft, Redmond, WA).

## RESULTS

To determine the mitotic role of Ran in vivo, we injected proteins that would perturb the Ran pathway into *Drosophila* embryos and assessed their effects on MT organization and karyokinesis. Early embryonic *Drosophila* nuclei are contained within a syncytial cytoplasm and undergo mitosis synchronously for the first 14 nuclear cycles, followed by cellularization (Foe and Alberts, 1983). At nuclear cycle 10, nuclei are at the embryo cortex where spindle assembly (Video 1) and nuclear division (Video 2) can be monitored by four-dimensional confocal microscopy. To reduce effects on Ran-dependent events during interphase (e.g., nuclear transport), embryos were injected just before mitotic entry, and cellular events were followed during the first mitosis after injection. However, it is still possible that a small percentage of defects arise from inhibiting Ran just before mitosis. Initially, when material is injected into an embryo, it will form a concentration gradient within the embryo with the highest concentration around the injection site. This concentration gradient of perturbant generates more severe effects proximal to the injection site and less severe effects distal to the injection site and has been described in other studies (Sharp *et al*., 2000a). This facet of the system results in different phenotypes depending on the amount of injection material reaching the spindle despite all the spindles sharing the same cytosol. This offers our study a significant advantage because we can see weak phenotypes that may be masked by more severe phenotypes when the Ran pathway is more severely inhibited.

### *Perturbing the Ran Pathway*

To perturb the Ran pathway, we injected recombinant proteins that are expected to either inhibit or activate the pathway. We used multiple strategies to inhibit the Ran pathway. One approach was to inject a dominant negative allele of Ran, RanT24N, which is locked in the GDP bound form (Kornbluth *et al*., 1994). RanT24N could inhibit the Ran pathway by binding to and inhibiting RCC1, the nuclear exchange factor that generates RanGTP (Dasso *et al*., 1994), and/or by binding to importin β (Hughes *et al*., 1998), although this binding is indirect and its affect on importin β function is unknown (Lounsbury *et al*., 1996). To gain insight

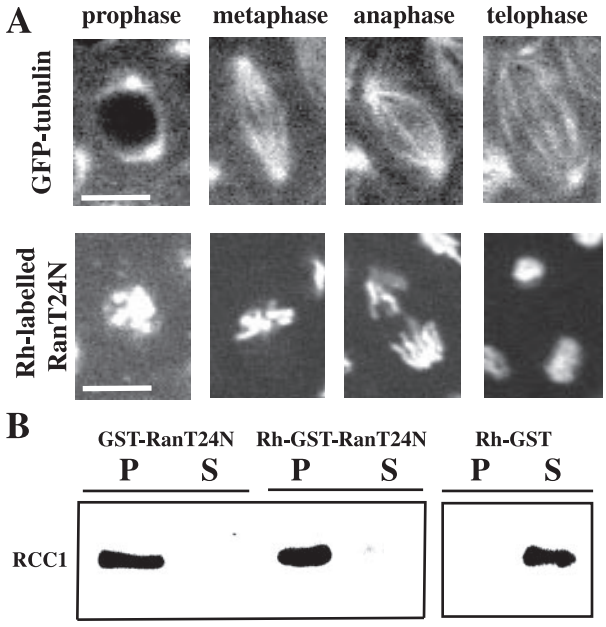


**Figure 1.** (A) Mitotic localization of injected rhodamine-labeled RanT24N in relation to spindle MT organization in a syncytial *Drosophila* embryo. (B) RCC1 binding assay. 6-His-RCC1 was incubated with glutathione agarose beads and either GST-RanT24N or rhodamine-labeled GST-RanT24N (Rh-GST-RanT24N), or rhodamine-labeled GST (Rh-GST). The beads were subsequently isolated, and the ability of 6-His-RCC1 to bind to the different GST fusion proteins (in the pellet fraction, P) or not bind (in the supernatant fraction, S) was assayed by immunoblotting and probing with an anti-6-His antibody. Bars, 10 $\mu$m.

into which inhibitory process predominates during mitosis in *Drosophila* embryos, we visualized the localization of rhodamine-labeled GST-RanT24N. Rhodamine-labeled RanT24N localized to condensed mitotic chromosomes throughout mitosis (Figure 1A), as does RCC1 (Trieselmann and Wilde, 2002). This localization pattern differs markedly from mitotic importin $\beta$, which localized throughout the embryo with some concentration at the residual nuclear envelope (Trieselmann and Wilde, 2002). Rhodamine-labeled GST-RanT24N binds equally well to RCC1 as unlabeled GST-RanT24N (Figure 1B). Thus, the predominant effect of injected RanT24N will likely be the inhibition of RCC1, thereby preventing the continual generation of RanGTP at chromosomes. In addition to RanT24N, the Ran pathway can be inhibited by injecting RanGAP, which activates the intrinsic GTPase activity of Ran, thus reducing the level of RanGTP in the embryo. This inhibition should be less severe than that obtained with RanT24N, because RanGAP does not affect the production of RanGTP.

In mitosis, the Ran pathway is proposed to function through RanGTP, which prevents the inhibition of NLS-containing SAFs via its NTR binding activity. Injecting RCC1 could elevate RanGTP levels, thereby up-regulating the pathway.

### *The Ran Pathway Is Required for Premetaphase Events*

***Ran Is Involved in Spindle Assembly.*** Inhibiting the Ran pathway in embryos disrupted spindle assembly (Figure 2, Supplemental Table S1, and Videos 3–4). As expected, injection of RanT24N caused the greatest frequency of severe spindle assembly defects (Figure 2B and Supplemental Table S1), compared with control injections of wild-type Ran and uninjected embryos where only minor effects on spindle assembly were seen (Figure 2B and Supplemental Table S1). The most dramatic phenotype was the complete inhibition of spindle assembly, where no spindle MTs were nucleated from either centrosomes or chromatin (Figure 2C). When MTs were observed, they emanated around condensed chromatin (Figure 2C) rather than from centrosomes as in control (Figure 2A) and failed to resolve into spindles. These MTs persisted around condensed chromatin, which stained positive for phospho-histone H3, suggesting that it remained in a mitotic state (Figure 2D). Although these effects were observed most frequently with the highest concentrations of RanT24N, they were also observed upon injection of RanGAP proximal to the injection site (Supplemental Table S1).

To observe more subtle effects of Ran, we injected lower concentrations of RanT24N and RanGAP that allowed some MT production. Under these conditions, spindles initially formed, but they soon became unstable and disorganized. Bundles of centrosomally nucleated MTs grew outward, away from the chromatin. Often, these MTs encountered MTs from neighboring spindles causing the spindles to fuse (Figure 2C and Video 3). Bipolar spindles that did form had altered dimensions (Figure 2E).

Spindle pole organization was also disrupted: poles became broader and less focused and in extreme cases, monopolar spindles formed (Figure 2C and Table S1). Where nuclei possessed multiple centrosomes, multipolar spindles formed (Figure 2C and Video 4). Sometimes, poles split or centrosomes were released (Figure 2C). Injection of recombinant RCC1 also disrupted spindles (Figure 2B and Table S1). Although many defects were similar to those caused by RanT24N, RCC1 did stimulate the formation of a higher proportion of multipolar spindles (Supplemental Table S1).

These data suggest that Ran is required for spindle assembly in vivo in *Drosophila* embryos.

***Nuclear Transport Receptors Inhibit Spindle Assembly.*** Previous studies strongly suggest that the mitotic function of Ran stems from RanGTP, generated at chromosomes, binding to NLS-containing SAFs and inhibiting them (reviewed in Di Fiore *et al*., 2004). Therefore, to determine whether this mechanism applies in vivo, we microinjected NTRs to elevate their levels within the embryo. These additional NTRs would be predicted to counteract the action of RanGTP and inhibit RanGTP-dependent events. One method was to inject recombinant nuclear import receptors either individually (importin $\alpha$ or importin $\beta$) or in combination, because they act together in a complex. In addition, we used a fragment of importin $\beta$ that lacks the amino-terminal domain, making it insensitive to RanGTP. Another way to elevate NTRs would be to inject RanBP1. RanBP1 can bind to RanGTP and stimulate the release of NTRs from Ran, in particular nuclear export factors and their cargo from RanGTP (Bischoff and Gorlich, 1997; Kehlenbach *et al*., 1999). Alternatively, RanBP1 could stimulate a decrease in RanGTP levels by enhancing RanGAP activity (Coutavas *et al*., 1993; Bischoff *et al*., 1995) or by inhibiting RCC1 (Nicolas *et al*., 1997). Although no homologue of RanBP1 exists in *Drosophila*, the Ran binding domain in human RanBP1 is 43.8% identical to those in *Drosophila* RanBP2 and would therefore act as a diffusible source of Ran binding domains to inhibit the Ran pathway.

Injection of NTRs affected spindles in a similar way as the injection of moderate concentrations of RanT24N, resulting in a comparable array and degree of phenotype severity (Figure 3, A and B, and Supplemental Table S1). These data suggest that Ran regulates spindle assembly through regulating the binding of NTRs to SAFs. Interestingly, upon

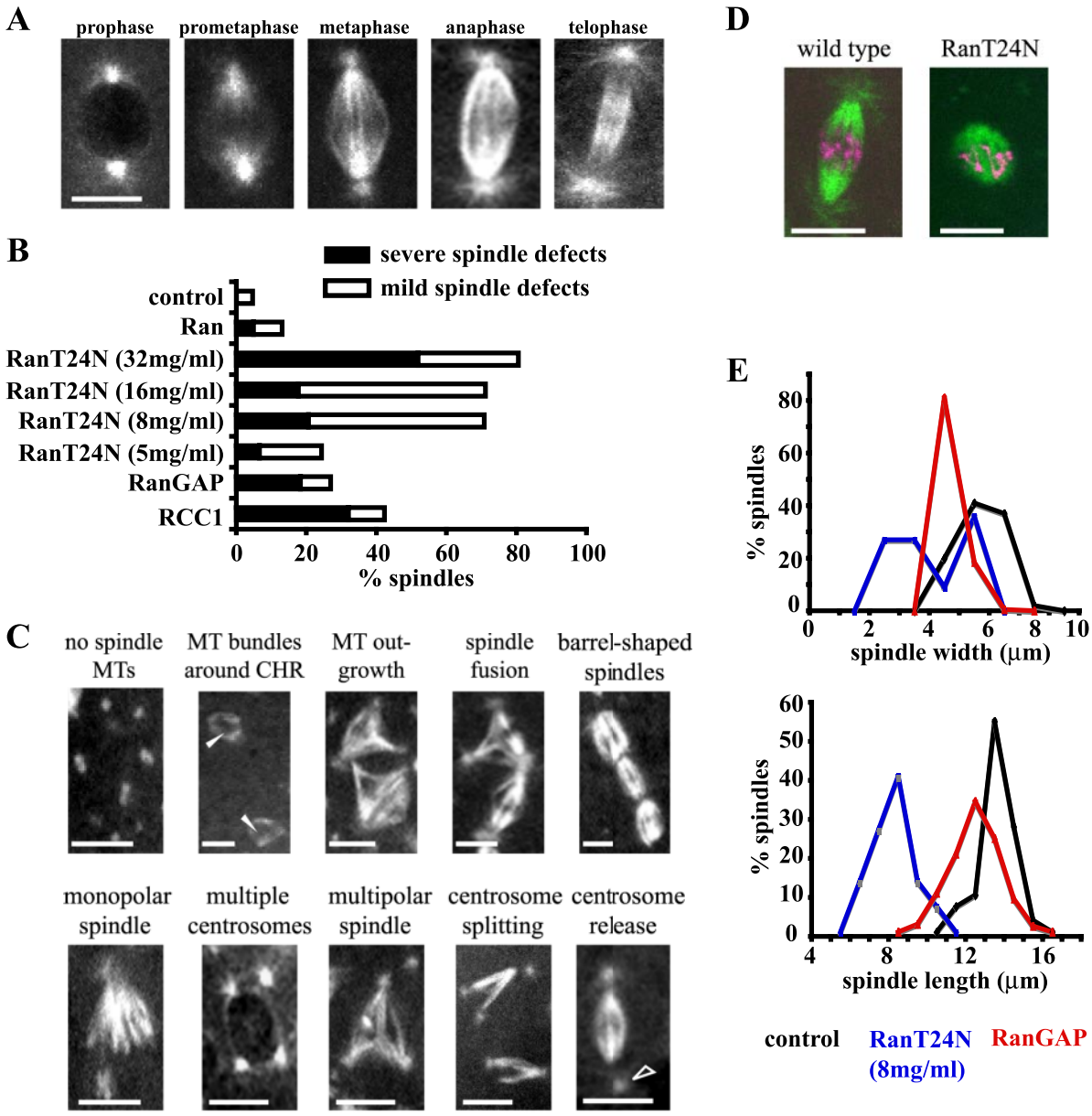


**Figure 2.** Perturbation of the Ran pathway disrupts spindle organization. (A) Spindle assembly in a control embryo expressing GFP-tubulin; images from a time-lapse series of Video 1. (B) Percentage of spindles disrupted upon perturbation of the Ran pathway. Severe defects: inhibition of spindle assembly, spindles form as bundles of MTs around chromosomes. Mild defects: spindle fusion, outward growth of MT, split poles, centrosome release, monopolar and multipolar spindles, narrow spindles, short spindles, spindles start to form around chromosomes, flat poles, "frozen" spindles. (C) MT organization after RanT24N injection. The solid arrowheads indicate chromatin and the open arrowheads indicate released centrosomes. CHR, chromosomes. (D) Micrographs of wild-type and RanT24N-injected embryos stained for tubulin (green), phospho-histone (red), and DNA (blue). (E) Distribution of spindle widths and lengths in cycle 12 embryos after disruption of the Ran pathway. Embryos injected with 8 mg/ml RanT24N (n = 170) and RanGAP (n = 378). Bars, 10 $\mu$m.

injection of pairs of importins, many more MTs initially formed in prometaphase as bundles around condensed chromosomes (Figure 3C, Supplemental Table S1, and Video 5a). In contrast, MTs in control embryos were nucleated from centrosomes and rapidly grew toward chromosomes (Figure 3C and Video 5b). In NTR-injected embryos, bundles of MTs slowly reorganized into spindle-like structures but never fully resolved into stable spindles.

Injection of RanBP1 also caused similar defects to injected RanT24N and NTRs (Figure 3, A and B, and Supplemental Table S1). However, uniquely some spindles exhibited consecutive defects. First centrosomes were released, then the spindle elongated (Figure 3D and Video 6). Because one function of RanBP1 is to stimulate the release of nuclear exported cargo from exportins on the cytoplasmic face of the nuclear pore complex (Kehlenbach *et al.*, 1999), we tested whether a nuclear exportin has a role in spindle assembly. Injection of CAS, the nuclear export factor for importin $\alpha$, resulted in defects similar to those caused by low doses of RanT24N (Figure 3A and Supplemental Table S1). However, there were differences: CAS caused the highest frequency of multipolar spindles (Supplemental Table S1).

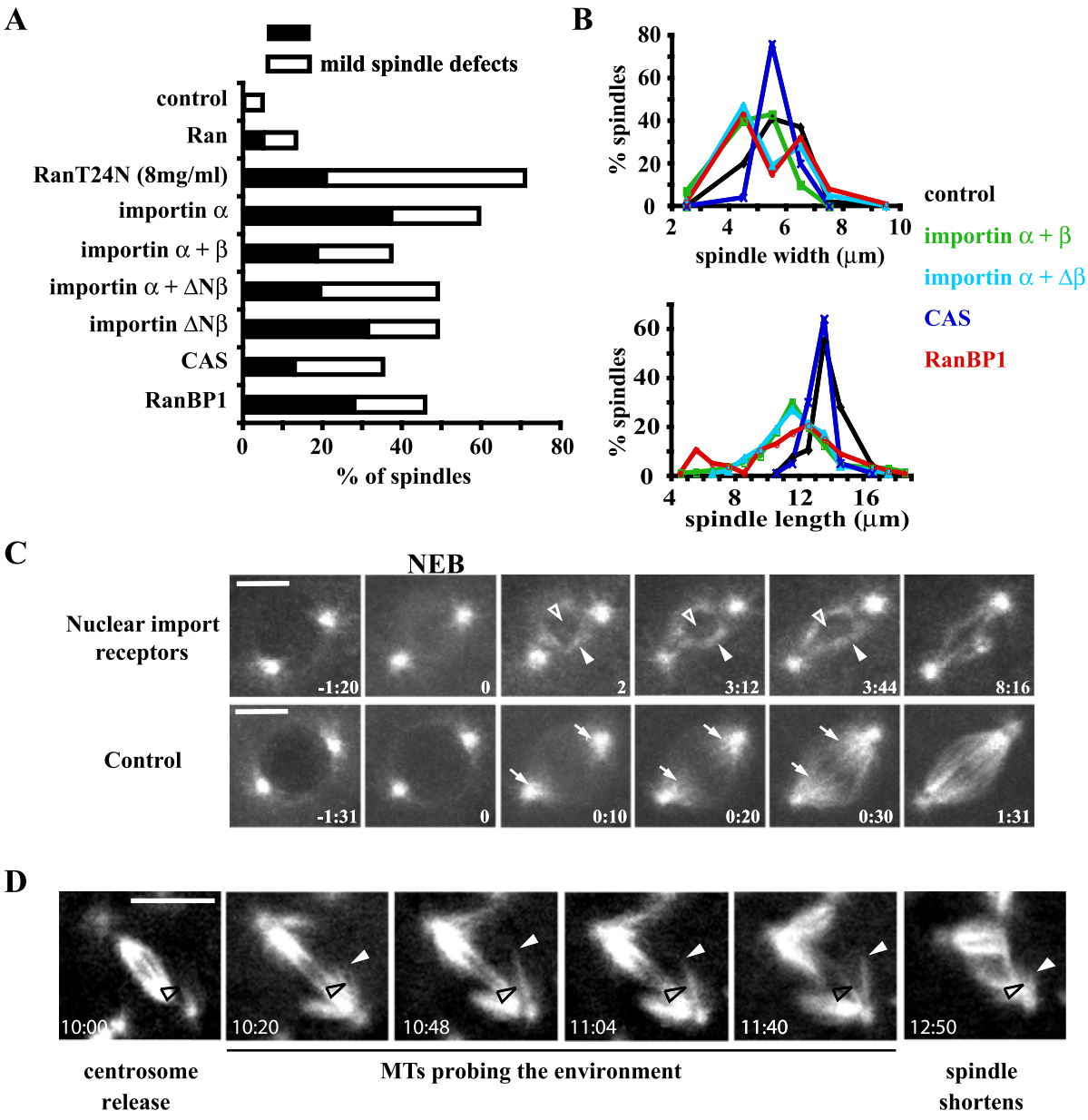


**Figure 3.** Elevated levels of NTRs disrupts spindle assembly. (A) Percentage of spindles disrupted upon perturbation of the Ran pathway. Severe defects: inhibition of spindle assembly, spindles form as bundles of MTs around chromosomes. Mild defects: spindle fusion, outward growth of MT, split poles, centrosome release, monopolar and multipolar spindles, narrow spindles, short spindles, spindles start to form around chromosomes, flat poles, "frozen" spindles. (B) Distribution of spindle widths and lengths in cycle 12 embryos after disruption of the Ran pathway. Embryos injected with importin $\alpha$ + importin $\beta$ (n = 230), importin $\alpha$ + $\Delta$N importin $\beta$ (n = 101), CAS (n = 250), and RanBP1 (n = 180). (C) MT organization in GFP-tubulin–expressing embryos injected with either importin $\alpha$ + $\Delta$N importin $\beta$ (Nuclear import receptors) or wild-type Ran (Control). Open arrowheads indicate chromatin position; solid arrowheads mark centrosome independent MT assembly around chromatin. Arrows indicate centrosomally nucleated MTs growing rapidly toward chromatin. Nuclear import receptor injected embryo images are taken from a time-lapse series of Video 5a. Control images are from a time-lapse series of Video 5b. Nuclear envelope breakdown (NEB) represents time 0 (minutes). (D) Time-lapse series of MT organization after injection of RanBP1. The open arrowheads mark the initial position of a MT tip and the solid arrowheads follow the MT tip probing the environment over time. Images from a time-lapse series of Video 6. Numbers represent time in minutes after injection. Bar, 10 $\mu$m.

Together, these data suggest that Ran, through its modulation of NTR binding to SAFs, regulates multiple aspects of spindle assembly in syncytial *Drosophila* embryos.

***MT Targeting to Chromosomes.*** A common phenotype seen in all injections that inhibit the Ran pathway (RanT24N, RanGAP, NTRs, and RanBP1) was a failure of centrosomally nucleated MTs to grow rapidly and directly toward chromosomes (Figure 3D and Video 6). On inhibition of the Ran pathway bundles of MTs, still focused at the poles began to grow in random directions, probing their environment by repeated cycles of growth and shrinkage (Figure 3D and Video 6). This is in stark contrast to control embryos where centrosomally nucleated MTs grew rapidly toward chromosomes (Figure 3C and Video 1). These data

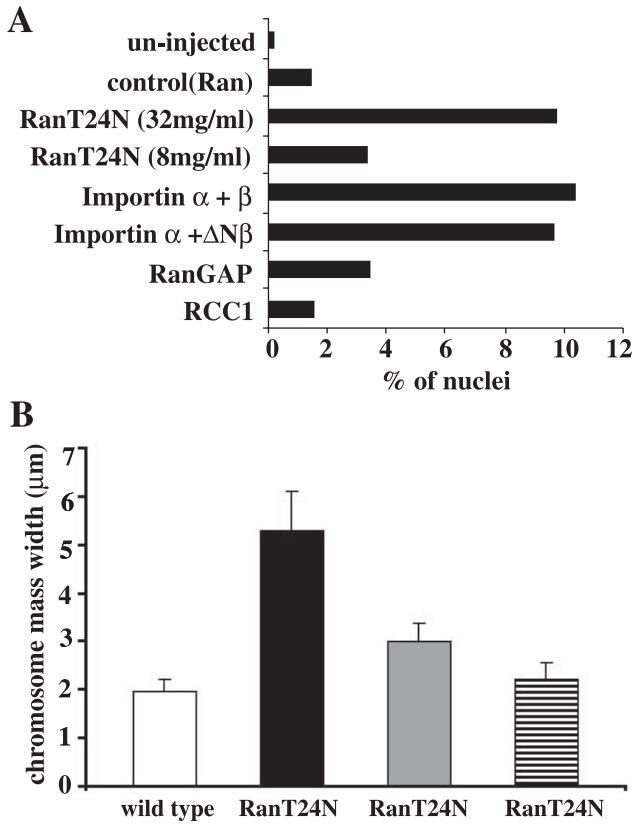


**Figure 4.** Ran pathway disruption inhibits chromosome condensation and metaphase plate formation. (A) Inhibition of chromosome condensation after disruption of Ran pathway. (B) Metaphase plate width of condensed mitotic chromosomes just before anaphase onset after injection of various concentrations of RanT24N. Error bars in B represent SD.

suggest that Ran is required for correct MT targeting to chromosomes by preventing NTRs binding to and inhibiting SAFs.

***Chromosome Organization.*** To analyze the role of the Ran pathway in mitotic chromosome organization, we used a similar strategy as described above using a *Drosophila* line expressing GFP-histone to visualize the chromatin. In a small number of nuclei, inhibiting the Ran pathway as described above prevented nuclear division (Figure 4A and Supplemental Table S2). Chromatin masses seemed to stay in an interphaselike state (decondensed) or early prophase-like state (slightly condensed) and did not divide, suggesting that either the Ran pathway has a role in chromosome condensation or Ran has a role in mitotic entry. Some nuclei persisted at the cortex, and others exhibited nuclear fallout (Supplemental Table S2). However, the DNA in most nuclei condensed and chromosomes segregated. In some cases, even where chromosomes condensed normally, congression to the metaphase plate was disrupted causing a broadening (~3-fold) of the metaphase plate in the centrosome-to-centrosome axis (Figure 4B and Table 1). Control injections of wild-type Ran had only minor effects on metaphase plate formation and nuclear division compared with uninjected embryos (Figure 4A and Table 1).

These data suggest that the Ran pathway is required for chromosome congression to the metaphase plate.

### *The Ran Pathway Is Required for Postmetaphase Events*

***Chromosome Movement and Segregation.*** Inhibiting the Ran pathway using factors that directly affect Ran (RanT24N, RanGAP, and RCC1) or downstream factors of Ran (NTRs), disrupted chromosome segregation to different degrees compared with uninjected and control-injected embryos (Figure 5 and Supplemental Table S2). Frequently, inhibition of the Ran pathway resulted in the formation of anaphase bridges. Although some bridges resolved (Figure 5C), others did not (Figure 5D), leading to the formation of nuclei with a 4N DNA content (judged by a doubling of the fluorescence intensity compared with adjacent nuclei that had normal division). In less severe cases, individual chromosomes lagged behind the main chromosome mass (Figure 5E, arrows). Occasionally, disjoined chromosomes fused with their sister chromosomes resulting in nuclei with a 4N DNA content. In extreme cases, separated chromosomes from two or more nuclei fused, leading to the formation of large chromosomal masses (Figure 5F, arrows, and Video 7) or two or more nuclei fused (Figure 5G, arrows).

Other chromosome segregation phenotypes stemmed from apparent defects in cell cycle progression as chromosomes remained in a metaphase-condensed state (Supplemental Table S2). Occasionally, there seemed to be an override of the spindle checkpoint, as metaphase chromosomes decondensed without disjunction and transited into an interphaselike state (Supplemental Table S2). This was also the

**Table 1.** Perturbation of the Ran pathway affects chromosome congression to metaphase plate and anaphase chromosome velocity

| | CHR mass width (Avg ± SD [μm]) | P (width) | Velocity to poles ± SD (μm/s) | P (velocity) | CHR pairs |
|---|---|---|---|---|---|
| Wild type | 1.98 ± 0.25 | | 0.087 ± 0.01 | | 15 |
| Control (Ran) | 2.09 ± 0.2 | 0.14 | 0.083 ± 0.01 | 0.3 | 23 |
| RanT24N, 32 mg/ml | 5.3 ± 0.82 | <0.001* | 0.042 ± 0.02 | <0.0001* | 10 |
| RanT24N, 16 mg/ml | 3 ± 0.4 | <0.001* | 0.074 ± 0.01 | 0.0007* | 12 |
| RanT24N, 8 mg/ml | 2.23 ± 0.35 | 0.11 | 0.08 ± 0.03 | 0.37 | 9 |
| Importin α + β | 2.17 ± 0.48 | 0.22 | 0.084 ± 0.02 | 0.52 | 17 |
| Importin α + ΔNβ | 2.19 ± 0.29 | 0.04* | 0.067 ± 0.01 | 0.0005* | 9 |
| RanGAP | 2.23 ± 0.38 | 0.08 | 0.07 ± 0.02 | 0.0075* | 11 |
| RCC1 | 2.11 ± 0.35 | 0.15 | 0.086 ± 0.02 | 0.73 | 16 |

P, probability that a parameter is the same as in wild-type embryos based on a Student's *t* test. Asterisk (*) indicates statistically significant difference.

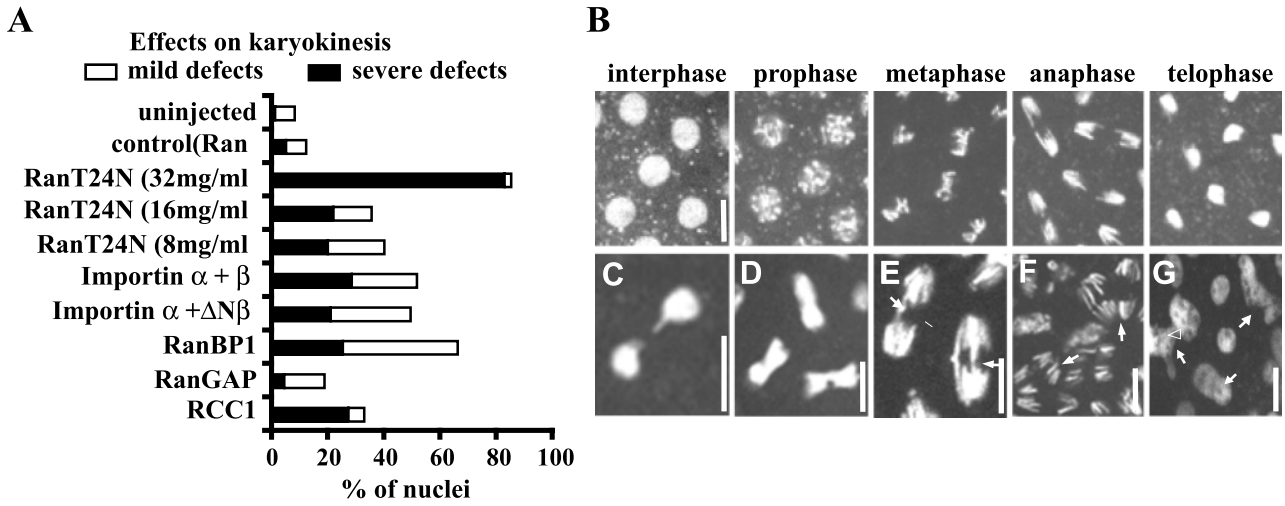


**Figure 5.** Perturbation of the Ran pathway disrupts chromosome segregation and chromosome movement. (A) Effects of Ran pathway disruption on chromosome segregation. Severe defects: metaphase arrest, decondensation of metaphase chromosomes, fusion of anaphase chromosomes from 1 nucleus, fusion of chromosomes from two nuclei, bridges that do not resolve, fusion of two nuclei, nuclear fallout. Mild defects: lagging chromosomes and bridges that resolve. (B) Nuclear division in a control embryo expressing GFP-histone; images from a time-lapse series of Video 2. (C–G) Nuclear division defects in embryos injected with RanT24N: bridges that resolve (C); bridges that do not resolve (D); lagging anaphase chromosomes (arrows) (E); fusion of chromosomes from adjacent nuclei (arrows) (F); image from a time-lapse series of Video 7; and fusion of nuclei (arrows) (G). Bars, 10 $\mu$m.

most common effect upon injection of RCC1, which should up-regulate the Ran pathway (Figure 5A, Supplemental Table S2, and Video 8).

Distal to the injection site on morphologically normal spindles inhibition of the Ran pathway caused up to a 50% reduction in the velocity of chromosome movement to the spindle poles (Table 1). In contrast, up-regulation of the pathway upon the injection of RCC1 did not alter the velocity of chromosome segregation.

Together, these data demonstrate that the Ran pathway is required for multiple steps in chromosome segregation during anaphase.

***Midbody Organization.*** The effect of inhibiting the Ran pathway was not restricted to spindle assembly. Distal to the injection site spindles formed normally and progressed to anaphase but were unable to form normal midbodies (Figure 6A). In some instances, midbody formation was completely inhibited, but when midbodies did assemble they had unbundled MTs (Figure 6, C, D, and E, i; and Video 9), were bent (Figure 6E, ii) or were narrow (Figure 6E, iii). Control injections of wild-type Ran had only minor effects on midbody organization compared with uninjected embryos (Figure 6A and Supplemental Table S3).

These data demonstrate a role for the Ran pathway after spindle assembly and metaphase, suggesting that the Ran pathway is required throughout mitosis for correct MT organization.

### *The Ran Pathway Is Required for Aurora A Targeting to Spindle MTs*

Perturbing the Ran pathway in vivo has dramatic effects on spindle assembly and function. Ran could achieve this by directly regulating the activity of a large number of SAFs, by regulating signal transduction pathways, or both. One candidate target is the Aurora A kinase, which in vitro is suggested to be downstream of Ran in the Ran spindle assembly pathway (Trieselmann *et al*., 2003; Tsai *et al*., 2003). Aurora A is recruited to centrosomes in interphase by centrosomin (Terada *et al*., 2003) where it is activated by ajuba (Hirota *et al*., 2003) and HEF-1 (Pugacheva and Golemis, 2005). During mitosis in somatic mammalian cells Aurora A relocates to spindle MTs in a TPX2-dependent manner (Kufer *et al*., 2002).

To address whether Aurora A could be affected by Ran, we assessed Aurora A localization in control and RanT24N-injected embryos. In control embryos, Aurora A localized to centrosomes in interphase and prophase, but in prometaphase it began to redistribute along spindle MTs (Figure 7A). On injection of RanT24N, 83.3% of spindles (n = 96) showed mislocalization of Aurora A, which now concentrated at centrosomes and did not localize to spindle MTs (Figure 7A).

To determine whether Aurora A targeting to MTs is regulated by NTRs as demonstrated in vitro (Trieselmann *et al*., 2003), we injected ΔN importin $\beta$ and analyzed Aurora A localization as described above. ΔN importin $\beta$ injection also prevented Aurora A redistribution to spindle MTs in 92% of spindles (n = 27) (Figure 7A). This strongly suggests that in vivo Aurora A function is regulated by a NTR sensitive SAF.

### *The Ran Pathway Is Required for Skeletor Organization*

We next tested whether the Ran pathway is required for MT-independent mitotic events. One such process is the assembly of a matrix as defined by one of its components, skeletor (Walker *et al*., 2000). RanT24N disrupted skeletor distribution within the embryo in 80.9% of spindles (n = 220). Skeletor was asymmetrically distributed outside the area of disrupted spindles (Figure 7B), persisting in the vicinity of condensed chromatin rather than around the residual spindle. These data suggest that skeletor organization is dependent upon the Ran pathway.

### *The Ran Pathway Is Required for the Localization of the Motors KLP61F and KLP3A*

Many of the spindle defects observed upon disruption of the Ran pathway could stem from misregulation of motor pro-

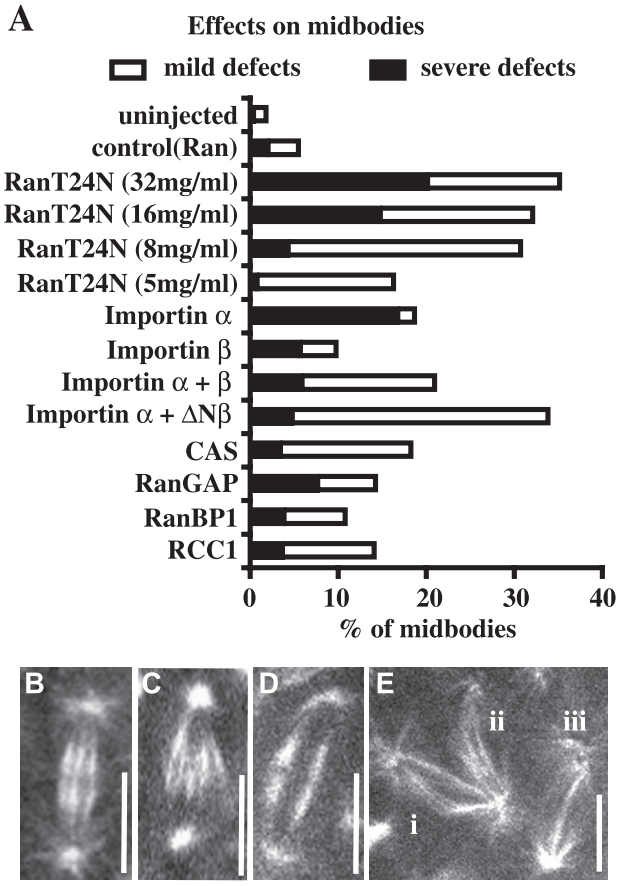


**Figure 6.** Perturbation of the Ran pathway disrupts midbody organization. (A) Percentage of midbodies disrupted upon perturbation of the Ran pathway. Severe defects: no midbodies and O-shaped midbodies. Mild defects: bent, split, laterally splayed, and very narrow midbodies. (B–E) Images from time-lapse series. (B) Midbody in a control *Drosophila* embryo expressing GFP-tubulin. (C–E) Midbody organization after RanT24N injection: split midbody (C); splayed midbody (D); O-shaped midbody (E, i); bent midbody (E, ii); and narrow midbody (E, iii). Bars, 10 μm.

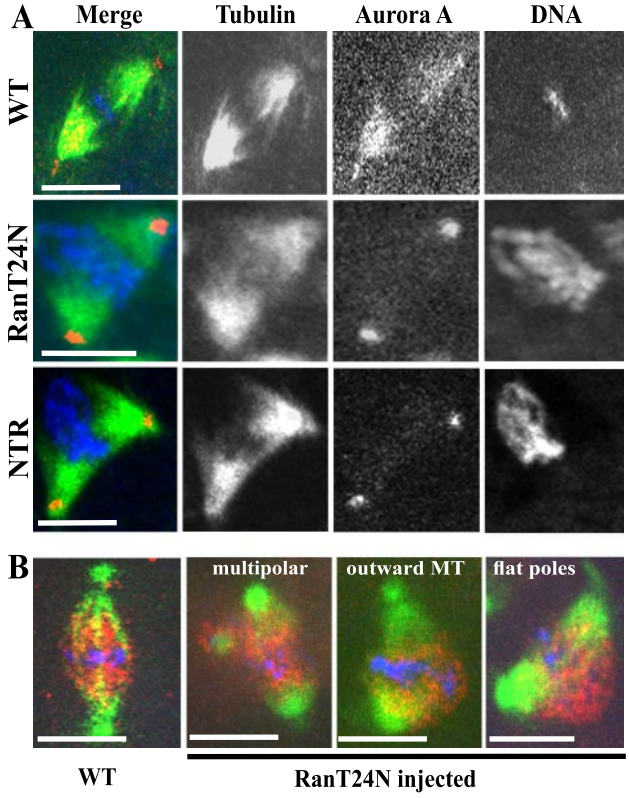


**Figure 7.** The Ran pathway is required for Aurora A targeting to spindle MTs and skeletor organization. (A) Aurora A distribution in spindles of wild-type, RanT24N, and NTR-injected embryos stained for tubulin (green), Aurora A (red), and DNA (blue). (B) Skeletor distribution in spindles of wild-type and RanT24N-injected embryos stained for tubulin (green), skeletor (red), and DNA (blue). Bars, 10 μm.

teins. Indeed, previous in vitro studies showed that the Ran pathway affects the function of Eg5, a tetrameric kinesin of the kinesin-5 family (Lawrence *et al*., 2004) involved in spindle assembly (Wilde *et al*., 2001), of Kid (Trieselmann *et al*., 2003), a chromokinesin of the kinesin-10 family (Lawrence *et al*., 2004), and XCTK2 (Ems-McClung *et al*., 2004), a mitotic kinesin of kinesin-14 family (Lawrence *et al*., 2004). Therefore, we examined the consequence of disrupting the Ran pathway on the localization of KLP61F (the *Drosophila* homologue of Eg5) and KLP3A, a *Drosophila* chromokinesin from the kinesin-4 family (Lawrence *et al*., 2004).

After the injection of RanT24N, KLP61F distribution was disrupted in 91.8% of spindles (n = 320) (Figure 8A). In control embryos, phospho-KLP61F (phosphorylated at T933 and the form of KLP61F that is recruited to spindles) (Sharp *et al*., 1999a) has a cell cycle-dependent distribution: it is nuclear in interphase and localizes to spindle MTs in prometaphase and metaphase, with a small proportion at centrosomes. In anaphase and telophase, KLP61F localizes to the central spindle (Sharp *et al*., 1999a). However, upon RanT24N injection KLP61F was barely detectable on spindles with disorganized MTs (Figure 8A, i). Furthermore, an absence of KLP61F staining at centrosomes correlated with metaphase spindles that were shorter and had broader poles (Figure 8A, ii) (n = 14). In addition, in RanT24N injected embryos KLP61F did not localize to 93.7% of midbodies (n = 32) (Figure 8B).

KLP3A has a cell cycle-dependent distribution localizing to the nucleus in interphase and relocating to the central spindle region, including chromosomes, from prometaphase to anaphase. In telophase, KLP3A persists on the central midbody MTs (Kwon *et al*., 2004). After injection of RanT24N, KLP3A distribution was disrupted in 96.2% of spindles (n = 80): KLP3A localized to chromosomes but not to central spindle MTs (Figure 8C). Together, these data demonstrate that one function of the Ran pathway is to regulate the targeting of mitotic motors in vivo.

## DISCUSSION

We have shown that in syncytial *Drosophila* embryos the Ran pathway is required throughout mitosis to regulate MT-dependent and MT-independent events.

### *Ran Regulates Centrosomal Spindle MT Production*

Our data suggest that centrosomal MT production is regulated by NTR-sensitive SAFs. Severe disruption of the Ran pathway by injection of high concentrations of RanT24N inhibited MT production at centrosomes. However, less severe disruption of Ran pathway led to MT production

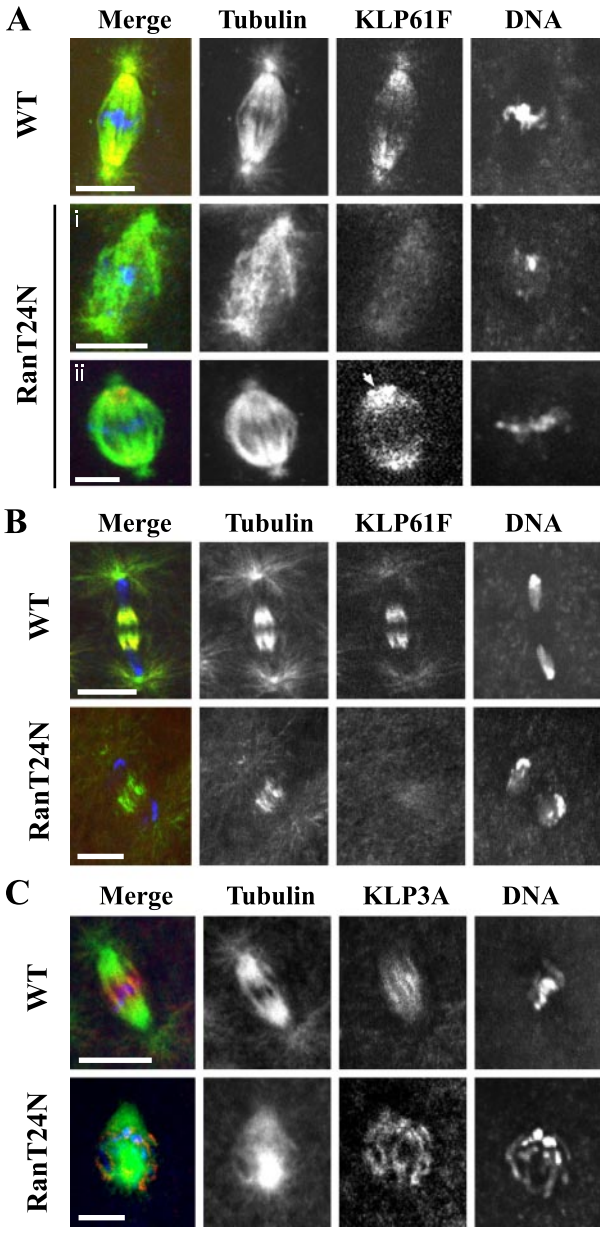


**Figure 8.** The Ran pathway is required for the correct localization of KLP61F and KLP3A. (A) KLP61F distribution in spindles of wild-type and RanT24N-injected embryos stained for tubulin (green), KLP61F (red), and DNA (blue). (i) Disrupted spindle MT organization. (ii) Spindle with broad poles; arrows indicate the absence of staining at centrosomes. (B) KLP61F distribution in midbodies of wild-type and RanT24N-injected embryos stained for tubulin (green), KLP61F (red), and DNA (blue). (C) KLP3A distribution in spindles in wild-type and RanT24N-injected embryos stained for tubulin (green), KLP3A (red), and DNA (blue). Bars, 10 $\mu$m.

around chromosomes. These MTs began to organize into a spindle in a manner reminiscent of acentrosomal spindle assembly in higher plants and female meiosis. In these conditions, it is possible that a small amount of RCC1 remained active and capable of producing a small amount of RanGTP. This reduced level of RanGTP may be sufficient to maintain active SAFs close to the chromatin and not around centrosomes some distance from the chromosomes. The MTs failed to resolve into stable spindles, possibly because of the rapid onset of the next interphase.

MTs nucleated from centrosomes when the Ran pathway was only partially inhibited (upon injection of lower concentrations of RanT24N, RanGAP, and NTRs) were short and failed to grow toward the chromosomes. These data suggest that the Ran pathway is required for the stabilization of centrosomal nucleated MTs in vivo, consistent with recent in vitro findings (Caudron *et al*., 2005). Thus, embryonic spindle assembly might be driven by both a dominant centrosomal-mediated pathway required for the rapid assembly of spindles and a backup chromosomal pathway as in mammalian cells (Tulu *et al*., 2003). The molecular targets of this pathway are unclear because TPX2 (Schatz *et al*., 2003) and XMAP310 (Anderson and Karsenti, 1997), which are required in vertebrates, have no known orthologues in *Drosophila*.

### *Ran Is Required for MT Targeting to Chromosomes*

Inhibition of the Ran pathway often caused centrosomally nucleated MTs to randomly probe their environment rather than grow rapidly toward chromosomes. Previous studies have implied that RanGTP complexed with importin $\beta$ persists around the spindle throughout mitosis (Kalab *et al*., 2002; Li and Zheng, 2004; Caudron *et al*., 2005). Therefore, RanGTP generated at chromosomes could form a spatial cue to guide MTs toward chromosomes. Our data support this hypothesis and suggest that MT targeting to chromosomes may not proceed by a random search and capture mechanism. Instead, a "directed" process operates where chromosomally generated RanGTP creates a molecular environment around chromosomes that facilitates MT growth preferentially toward the chromosomes. Such a scenario has recently been predicted in a mathematical model (Wollman *et al*., 2005).

### *Ran Regulates Spindle Pole Organization*

Our study showed that the Ran pathway has a role in spindle pole/centrosome regulation in vivo as perturbation of the Ran pathway caused defects in spindle pole and centrosome organization. When the Ran pathway was disrupted centrosomes were released, suggesting that the Ran pathway regulates factors required for centrosome attachment to spindle poles. Perturbation of the Ran pathway also led to the formation of multipolar spindles (Figure 2B), sometimes by the splitting of existing poles (Figure 2B). Our findings are consistent with previous studies in which overexpression of RanBP1 led to centrosome abnormalities (Di Fiore *et al*., 2003).

The mechanism by which Ran regulates the spindle pole/centrosome remains unclear. However, many of the effects have been described upon inhibition of dynein (Robinson *et al*., 1999, Sharp *et al*., 2000a) and Aurora A (Marumoto *et al*., 2003). Aurora A has been shown to be regulated by the Ran pathway in vitro requiring RanGTP for its targeting and activation (Trieselmann *et al*., 2003; Tsai *et al*., 2003; our study). Whether dynein or a regulator of dynein function is regulated by Ran remains to be tested. However, Ran itself may directly affect centrosomes because it has been localized to centrosomes through an interaction with AKAP450 (Keryer *et al*., 2003, Di Fiore *et al*., 2004). Interestingly, we saw multipolar spindles form upon injection of RCC1, suggesting that elevated levels of RanGTP also disrupt this process.

In addition, the failure of KLP61F to be recruited to centrosomes correlated with shorter spindles with broad poles. Previous studies that directly inhibit KLP61F have not pointed to a role in these processes (Sharp *et al*., 1999b). However, because our experiments inhibit a broad range of processes that are

downstream of Ran, it suggests that either a subset of KLP61F regulators, or factors working in coordination with KLP61F, may have a combined role in pole organization (see later discussion).

### *Ran Is Required for Spindle MT Organization*

The Ran pathway is required for the organization of spindle MTs. Strikingly, the disorganization did not result in a single spindle phenotype (Supplemental Table S1), suggesting that in *Drosophila* embryos, Ran regulates multiple factors involved in spindle organization. Many defects were consistent with a disruption of the balance of forces generated by antagonistic motors that are crucial for spindle assembly (Sharp *et al*., 1999b). Indeed, we show that KLP61F and KLP3A, two motors that operate in balance with other motors, require the Ran pathway for their correct localization to the spindle. The spindle phenotypes observed do not correspond to just the inhibition of KLP61F. This could be due to the motor that operates in balance with KLP61F, Ncd, also being regulated by the Ran pathway. Indeed, the *Xenopus* homologue of Ncd, XCTK2 has been shown in vitro to be regulated by the Ran pathway (Ems-McClung *et al*., 2004). Inhibition of both motors in *Drosophila* embryos (Sharp *et al*., 1999b; Sharp *et al*., 2000b) or of homologues of both motors in mammalian system (Mountain *et al*., 1999) largely cancels out the individual phenotypes. The phenotypes that are seen upon inhibition of these two motors in *Drosophila* embryos (Sharp *et al*., 1999b) we see upon perturbing the Ran pathway. The disruption of a balance of forces is unlikely to occur uniformly throughout an embryo due to the gradual diffusion of the injected material and would therefore result in a variety of spindle organization defects.

### *Ran Is Required for Chromosome Congression*

Injection of RanT24N caused the metaphase plate to be three times wider (when spindle assembly proceeded) than in controls. A widening of the metaphase plate could be due to defects in MT attachment to kinetochores or inhibition of chromokinesins, proteins that generate the "polar ejection force" that move chromosomes to the metaphase plate. Recently, the Ran pathway was shown to affect MT attachment to kinetochores (Arnaoutov *et al*., 2005). We showed that the Ran pathway can regulate chromosome congression by regulating the MT attachment of KLP3A (a Kinesin-4 family member) reminiscent of regulation by Ran of the chromokinesin Kid (a Kinesin-10 family member) (Trieselmann *et al*., 2003). These findings suggest that chromokinesins from different kinesin families may share a common regulatory mechanism. The role of Ran in chromosome congression may not be restricted to the direct regulation of MT kinetochore attachment and chromokinesins because Aurora A, which is also regulated by Ran (see below), is required for chromosome alignment at the metaphase plate in vertebrates (Marumoto *et al*., 2003).

### *Ran Is Required for Chromosome Segregation*

Anaphase A chromosome movement to the poles was slower in nuclei that otherwise progressed normally through mitosis upon Ran pathway perturbation. The reduced chromosome to pole velocity (Table 1) is identical to chromosome velocity in KLP59C-inhibited embryos (Rogers *et al*., 2004). KLP59C, a kinetochore-localized MT depolymerase of the Kinesin-13 family is not an obvious candidate to be regulated by Ran, because it is cytosolic (Mennella *et al*., 2005). Therefore, either a regulator of KLP59C or a protein that works in coordination with KLP59C, such as dynein, could be a target of the Ran pathway. Indeed, dynein inhibition causes similar reductions in chromosome movement to the poles. Another potential candidate is KLP3A (Kwon *et al*., 2004), which is involved in chromosome segregation and whose targeting is dependent upon the Ran pathway (this study).

Nuclear divisions were also disrupted upon RCC1 injection, resulting in chromosome decondensation without chromosome disjunction. This could be caused by a chromosome decondesation defect or an override of the spindle checkpoint. The latter would be consistent with a study in *Xenopus* egg extracts, where increased levels of RCC1 circumvented the spindle checkpoint by disrupting the localization of checkpoint regulators (Arnaoutov and Dasso, 2003).

### *Ran Is Required for Midbody Organization*

Disruption of the Ran pathway prevented the correct assembly of the midbody in morphologically normal spindles. A failure to bundle midbody MTs was the most common defect. Bundling of midbody MTs requires the action of multiple kinesins, including Ncd (Sharp *et al*., 1999a), KLP3A (Kwon *et al*., 2004), KLP61F (Sharp *et al*., 1999a), and Pavarotti (Adams *et al*., 1998). Indeed, we show that KLP61F fails to localize to midbodies in embryos where the Ran pathway is perturbed. In extreme cases, no midbody MTs formed, suggesting that the Ran pathway, either through MT stabilizing factors or through regulating MT bundling, is required to for midbody stability.

### *Ran Regulates Aurora A Targeting to Spindles*

In vitro biochemical assays have shown that Aurora A activation is a downstream event of the mitotic Ran pathway (Trieselmann *et al*., 2003; Tsai *et al*., 2003). Injection of both RanT24N and $\Delta$N importin $\beta$ caused similar defects in Aurora A localization; suggesting that in vivo the Ran pathway via importins regulates Aurora A by facilitating its targeting to spindle MTs. Therefore, regulating the targeting and subsequent activation of Aurora A could be a major in vivo role for the Ran pathway in coordinating spindle assembly, especially because Aurora A has been implicated in both pre- and postmetaphase mitotic processes (Marumoto *et al*., 2003).

Together, our data suggest that the Ran pathway has to remain active postmetaphase for chromosomes to successfully segregate. Indeed, we find that proteins expected to increase RanGTP levels (RCC1) and those expected to decrease RanGTP levels (RanT24N and RanGAP) affect mitosis, suggesting that a balance of RanGTP/GDP may be crucial for mitotic progression. Our data also suggests that a major mitotic role for Ran in *Drosophila* embryos is to prevent NTRs inhibiting SAFs during mitosis. Furthermore, we demonstrate that the mitotic role of the Ran pathway is not restricted to MT-dependent events as skeletor organization, which is independent of MTs, is dependent upon Ran. Therefore, the Ran pathway has the potential to regulate mitosis to a far greater extent than previously thought.

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