A Ran signalling pathway mediated by the mitotic kinase Aurora A in spindle assembly

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Published online: 10 February 2003; DOI: 10.1038/ncb936

The activated form of Ran (Ran-GTP) stimulates spindle assembly in *Xenopus laevis* egg extracts, presumably by releasing spindle assembly factors, such as TPX2 (target protein for Xenopus kinesin-like protein 2) and NuMA (nuclear-mitotic apparatus protein) from the inhibitory binding of importin-α and -β. We report here that Ran-GTP stimulates the interaction between TPX2 and the *Xenopus* Aurora A kinase, Eg2. This interaction causes TPX2 to stimulate both the phosphorylation and the kinase activity of Eg2 in a microtubule-dependent manner. We show that TPX2 and microtubules promote phosphorylation of Eg2 by preventing phosphatase I (PPI)-induced dephosphorylation. Activation of Eg2 by TPX2 and microtubules is inhibited by importin-α and -β, although this inhibition is overcome by Ran-GTP both in the egg extracts and *in vitro* with purified proteins. As the phosphorylation of Eg2 stimulated by the Ran-GTP–TPX2 pathway is essential for spindle assembly, we hypothesize that the Ran-GTP gradient established by the condensed chromosomes is translated into the Aurora A kinase gradient on the microtubules to regulate spindle assembly and dynamics.

The assembly of meiotic or mitotic spindles is one of the most complex processes in eukaryotic cells. It involves microtubules, microtubule motors and non-motor microtubule-associated proteins, which are coordinately regulated by protein kinases and phosphatases[1,2]. The mitotic kinase Aurora A[3] is essential for establishing and/or maintaining the bipolar spindle[4–8]. Phosphorylation of Thr 288 in the activation loop of human Aurora A is essential for its kinase activity[9]. Furthermore, the kinase activity of Aurora A is required for proper spindle assembly in *Drosophila melanogaster*[10] and in tissue culture cells[11]. A number of studies have shown that activation of Aurora A kinase is negatively regulated by PPI (refs 9, 11–13). Moreover, the balance between the kinase activity of Aurora A and the phosphatase activity of PPI has been shown to be important for several mitotic and meiotic processes, including spindle assembly[11,14–16]. Whereas the essential roles of Aurora A and PPI in mitosis and meiosis have been well established, how the kinase and phosphatase are regulated is unknown.

Ran, a GTPase required for interphase nucleocytoplasmic trafficking, can stimulate spindle assembly in *Xenopus* egg extracts[17–20]. Recently, the role of Ran-GTP in spindle assembly was also established *in vivo*[21,22]. Initial studies using *Xenopus* egg extracts have shown that spindle assembly stimulated by Ran-GTP requires proteins that mediate microtubule polymerization and organization[17]. Detailed analyses showed that Ran-GTP regulates microtubule dynamics and nucleation[23,24]. Furthermore, Ran-GTP stimulates bipolar spindle organization by indirectly activating Eg5, a plus-end-directed kinesin that is essential for spindle assembly[23].

Two components of the spindle apparatus, TPX2 (refs 25, 26) and NuMA[27–29], were recently identified as potential downstream targets of Ran-GTP[30–32]. Both proteins interact with the nuclear import receptor importin-β and the binding of Ran-GTP to importin-β disrupts the interaction. As importin-α and -β are potent inhibitors of spindle assembly, it was hypothesized that Ran-GTP stimulates spindle assembly by releasing spindle assembly factors from the inhibitory binding of importin-α and -β[30–32]. Although the idea that Ran-GTP releases TPX2 and NuMA from the inhibitory binding of importin-α and -β is appealing, it is unclear how importin-α and -β inhibit the function of TPX2 and NuMA in spindle assembly.

As both Ran-GTP and Aurora A kinase are essential for spindle assembly[4–8,17–20], we reasoned that Aurora A and Ran-GTP might function in the same pathway. To test this, we used the *Xenopus* cytostatic factor (CSF)-arrested egg extract to assay whether Ran-GTP could stimulate the kinase activity of the *Xenopus* Aurora A, called Eg2. We immunoprecipitated Eg2 with three different antibodies from *Xenopus* egg extracts[33] treated with either Ran^{L43E} (an activated GTP-bound allele of Ran) or Ran^{T24N} (a dominant-negative form of Ran that mimics Ran-GDP)[17]. The immunoprecipitates were then assayed for kinase activity in the presence of γ-^{32}P-ATP, fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and analysed by autoradiography. Quantification by western blotting demonstrated that equivalent amount of Eg2 was immunoprecipitated (Fig. 1a). We found that Ran^{L43E} consistently stimulated the phosphorylation of a protein with a relative molecular mass (M_r) of ~100,000 (100K) in the immunoprecipitates (Fig. 1a). Therefore, Ran-GTP seems to stimulate Eg2 to phosphorylate a 100K protein.

As TPX2, the downstream target of Ran-GTP, is approximately 100K, we asked whether the 100K phosphorylated protein present in the above kinase assays is TPX2. Thus, we first made a glutathione *S*-transferase (GST)–TPX2 fusion protein and raised a

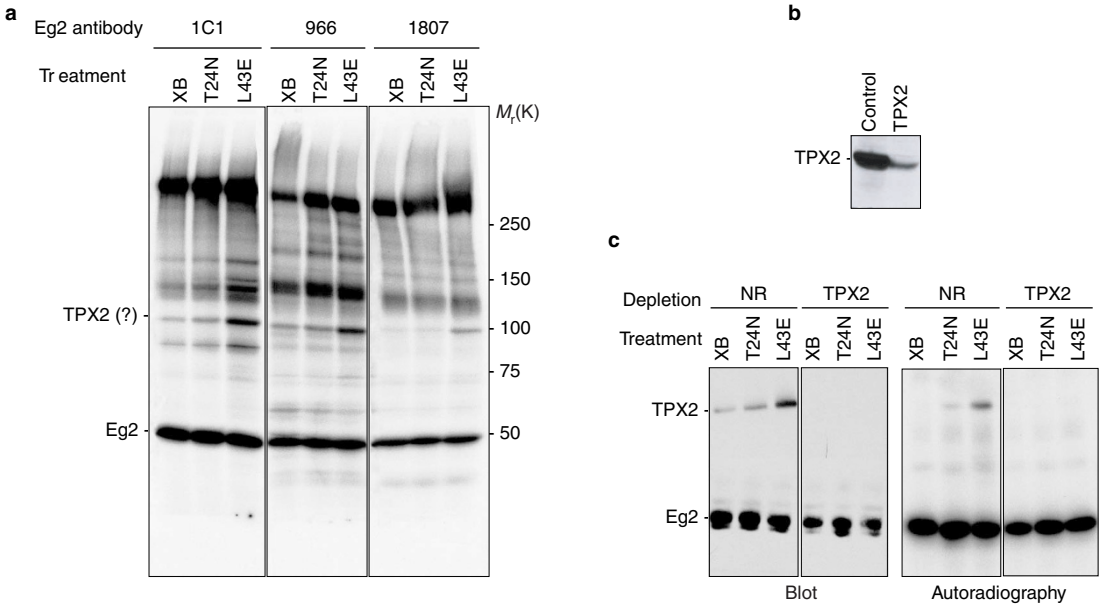

Figure 1 **Ran-GTP stimulates the interaction between TPX2 and Eg2.** **a**, Three different antibodies against Eg2 (1C1, 966 and 1807) were used to immunoprecipitate Eg2 from CSF egg extract supplemented with XB buffer (XB), Ran^{T24N} or Ran^{L43E}, before analysis in an *in vitro* kinase assay. Phosphorylated proteins were analysed by SDS–PAGE and autoradiography. Phosphorylation of a ~100K protein was enhanced by the presence of Ran^{L43E} in all three immunoprecipitations. **b**, TPX2 was depleted from the egg extract using an antibody against TPX2. Between 80 and 90% of TPX2 was depleted from the egg extract. Control extracts were mock-depleted with normal rabbit IgG. **c**, The depleted egg extracts were used to immunoprecipitate Eg2 using the 1C1 antibody before analysis by *in vitro* kinase assay. Samples were analysed either by western blotting probing with antibodies against TPX2 and Eg2, or by autoradiography.

rabbit polyclonal antibody against TPX2. Next, we immunodepleted TPX2 from the egg extracts using the affinity purified TPX2 antibody. Western blotting showed that 80–90% of the TPX2 in egg extracts was removed by the immunodepletion (Fig. 1b). The Eg2 immunoprecipitation/kinase assays were performed using the TPX2-depleted or mock-depleted egg extracts. Western blotting and autoradiography of mock-depleted extracts demonstrated that the 100K phosphorylated protein comigrated with TPX2 (Fig. 1c). Furthermore, removing TPX2 resulted in the loss of the 100K phosphorylated protein. From these observations, we conclude that the 100K band is TPX2. As more TPX2 is co-immunoprecipitated with Eg2 in the presence of Ran^{L43E}, compared with controls (Fig. 1c), Ran^{L43E} stimulates the interaction between Eg2 and TPX2 in *Xenopus* egg extracts, and this interaction in turn results in increased phosphorylation of TPX2.

Previous studies have shown that the kinase activity of human Aurora A depends on phosphorylation of Thr 288 (ref. 9). One possibility is that the binding of TPX2 to Eg2 could stimulate the phosphorylation, and thus activation, of Eg2. Therefore, we examined the effect on Eg2 of increasing the TPX2 concentration in the egg extract. Buffer, GST, GST–TPX2 or TPX2 were added into the egg extracts. Eg2 was then immunoprecipitated with the anti-Eg2 antibody 1C1 and analysed by western blotting using a rabbit polyclonal antibody against Eg2. We found that the migration of Eg2 was retarded in extracts treated with TPX2 when compared with controls (Fig. 2a, top), suggesting that TPX2 regulates the phosphorylation state of Eg2. To determine whether TPX2 stimulates phosphorylation, and thus activation of, Eg2, we generated phosphopeptide antibodies (P-Eg2) specific for the region of Eg2 that includes Thr 295, which is equivalent to Thr 288 in human Aurora A kinase. Phosphorylation of Thr 288 is known to activate the kinase activity of Aurora A. We repeated the above experiments and found that the phosphospecific antibody recognized Eg2 strongly in the TPX2-treated egg extracts when compared with mock-treated extracts (Fig. 2a, bottom). These observations demonstrate that in *Xenopus* egg extracts, TPX2 stimulates phosphorylation of Eg2 at Thr 295.

Previous studies have shown that human Aurora A and *Xenopus* Eg2 expressed in baculovirus have little kinase activity[9,13]. We expressed and purified histidine-tagged Eg2 using baculovirus[13] and confirmed that Eg2 was not phosphorylated at Thr 295 and that it had no kinase activity towards myelin basic protein (MBP), a commonly used substrate for this kinase (Fig. 2b). Therefore, we used the inactive baculovirus-expressed Eg2 to study whether TPX2 could activate Eg2 *in vitro*. We combined purified TPX2 and baculovirus-expressed Eg2 *in vitro*. Consistent with a recent report, we found that TPX2 interacts directly with Eg2 (ref. 34; data not shown). However, TPX2 alone neither stimulated Eg2 phosphorylation on Thr 295 nor Eg2 kinase activity towards MBP *in vitro* (Fig. 2b). As TPX2 and Eg2 colocalize on the mitotic spindle[25,34], we reasoned that microtubules might be required for activation of Eg2 by TPX2. To test this, baculovirus-expressed Eg2 was treated with purified TPX2 in the presence of taxol-stabilized microtubules. Western blotting showed that Eg2 became phosphorylated at Thr 295 in the presence of TPX2 and microtubules, but not in the presence of microtubules alone. Moreover, phosphorylated Eg2 could phosphorylate MBP (Fig. 2b). Therefore, TPX2 directly stimulates Eg2 phosphorylation and kinase activity in a microtubule-dependent manner. Next, we added TPX2 to the egg extracts in the presence or absence of nocodazole, a chemical that depolymerizes microtubules stimulated by excess TPX2 in the egg extracts[25,30]. We found that TPX2 failed to stimulate Eg2 phosphorylation in the presence of nocodazole (Fig. 2c). Therefore, TPX2-stimulated phosphorylation of Eg2 also requires the presence of microtubules in the egg extract.

We next asked how TPX2 and microtubules stimulate Eg2 kinase activity. Although baculovirus-expressed Eg2 is un-phosphorylated and inactive, previous studies have shown that Eg2 expressed in bacteria is active[7,8]. We examined whether Eg2 expressed in *Escherichia coli* was phosphorylated at Thr 295. As a control, a bacterially expressed 'kinase-dead' Eg2 ($Eg2^{K169R}$) that has

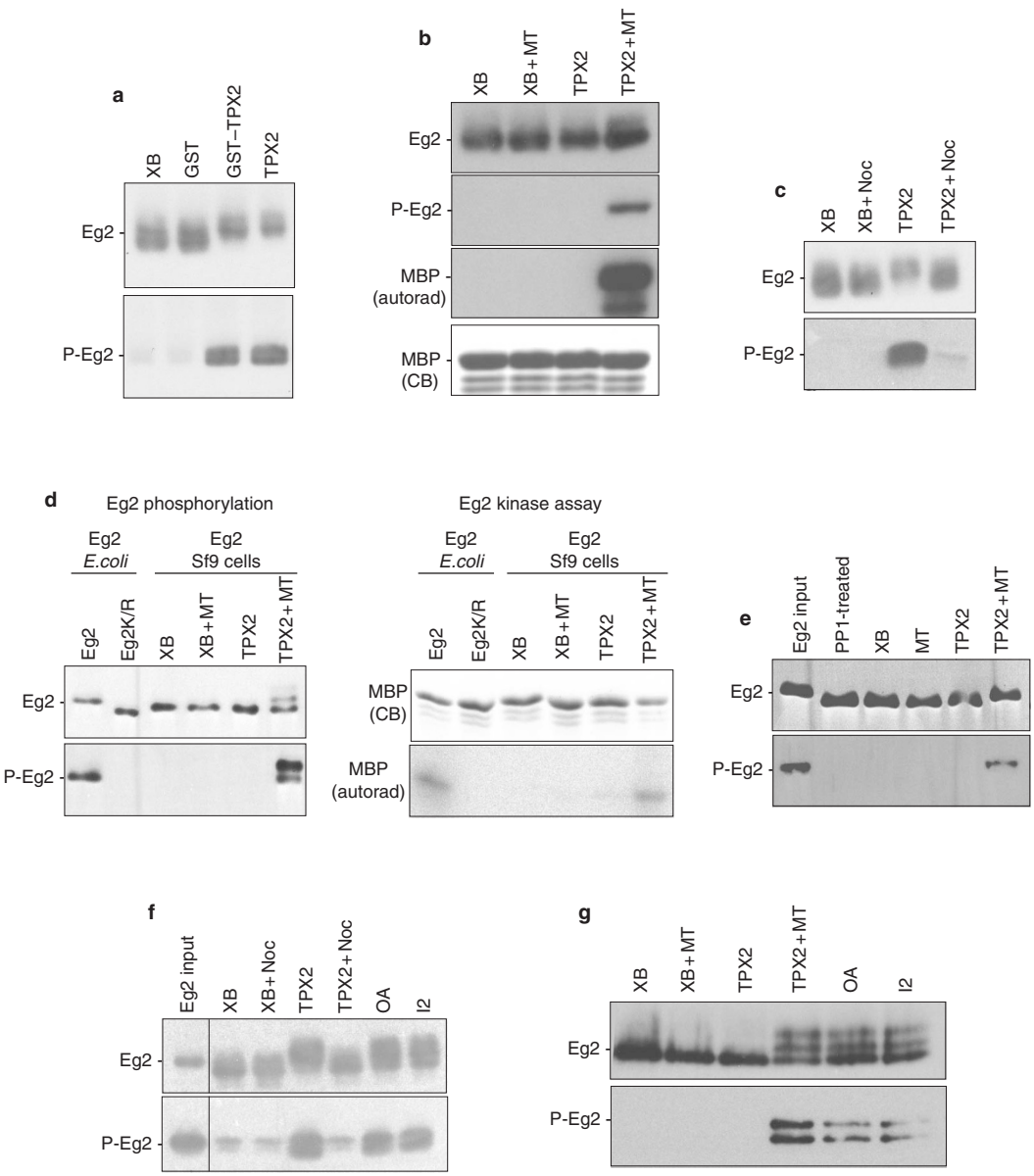

Figure 2 **TPX2 and microtubules stimulate phosphorylation of Eg2.** **a**, Egg extracts were treated for 10 min at room temperature, as indicated. Eg2 was immunoprecipitated with the 1C1 antibody before western blotting with an anti-Eg2 polyclonal antibody (top) or a phosphospecific antibody that recognizes P-Thr 295 of Eg2 (bottom). **b**, TPX2-mediated activation of Eg2 *in vitro* requires microtubules. Purified and baculovirus-expressed Eg2 (1 μM) was incubated with XB buffer, microtubules (10 μM tubulin) and/or TPX2 (0.5 μM) for 10 min at room temperature. Eg2 was either analysed by western blotting or used to phosphorylate MBP, which was then separated by SDS–PAGE and analysed by staining with Coomassie Blue (CB) and autoradiography. **c**, TPX2-stimulated phosphorylation of Eg2 requires microtubules in the egg extract. XB buffer or TPX2 was added to the egg extracts supplemented with nocodazole (Noc). Eg2 was analysed by immunoprecipitation and western blotting. **d**, Bacterially expressed wild-type Eg2, but not kinase-dead Eg2K/R is recognized by the phospho-specific antibody. However, baculovirus-expressed Eg2 is only phosphorylated after treatment with TPX2 and microtubules. MBP was used to assay the activity of bacterially expressed Eg2, Eg2K/R, or baculovirus-expressed Eg2 treated with buffer, microtubules and/or TPX2. **e**, In the presence of PPI, phosphorylation of bacterially expressed Eg2 becomes dependent on TPX2 and microtubules. Bacterially expressed Eg2 was treated by PPI before incubation with XB buffer, microtubules and/or TPX2. Eg2 was analysed by western blotting. **f**, TPX2 and microtubules activate Eg2 by counteracting PPI in the egg extracts. Bacterially expressed Eg2–His was added to extract supplemented with XB buffer, nocodazole, TPX2, OA or I2. After incubation, Eg2–His was isolated using nickel–agarose beads before western blotting. **g**, TPX2 and microtubules activate baculovirus-expressed Eg2 by counteracting PPI. Purified baculovirus-expressed Eg2 was incubated with XB buffer, microtubules, TPX2, OA or I2. Phosphorylation of Eg2 was determined by western blotting.

a mutation in the ATP-binding pocket, was used. Western blotting showed that bacterially expressed Eg2, but not $Eg2^{K169R}$ or baculovirus-expressed Eg2, was phosphorylated on Thr 295 and had kinase activity towards MBP (Fig. 2d). Addition of TPX2 and microtubules to bacterially expressed wild-type Eg2 or $Eg2^{K169R}$ did not stimulate phosphorylation or kinase activity (data not shown). However, baculovirus-expressed Eg2 required TPX2 and microtubules for its phosphorylation and kinase activity (Fig. 2d). This suggests that although TPX2 and microtubules stimulated baculovirus-expressed Eg2, they had no effect on the bacterially

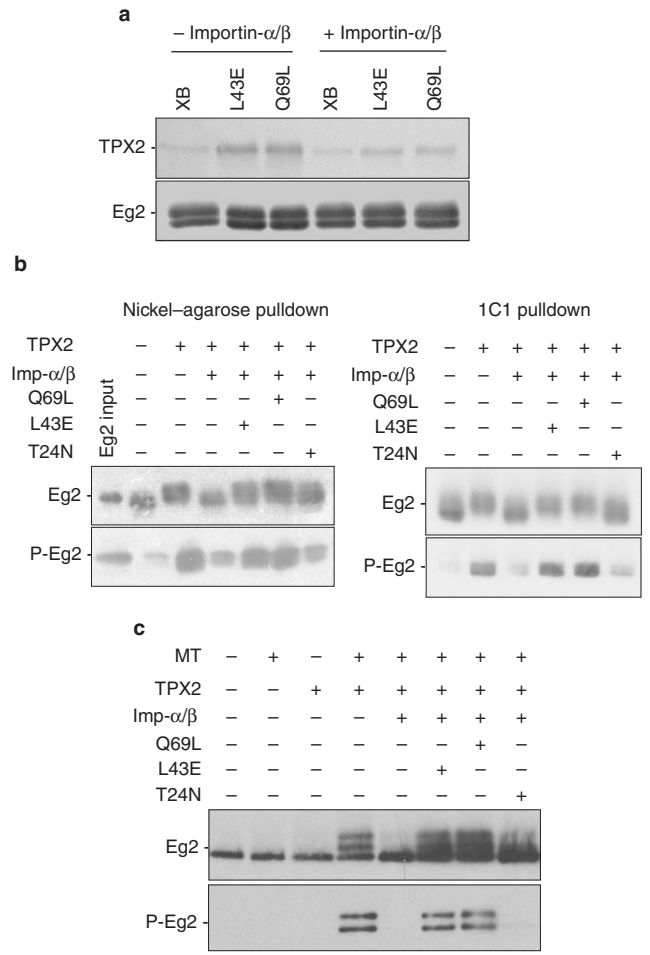

Figure 3 **TPX2-induced Eg2 phosphorylation is regulated by the Ran pathway. a**, Exogenously added importin-α/β inhibits the interaction between TPX2 and Eg2 that is enhanced by Ran. CSF egg extracts were treated with XB buffer, Ran^{L43E} or Ran^{Q69L} in the absence (left) or presence (right) of exogenous importin-α/β, before immunoprecipitation with the 1C1 antibody. Immunoprecipitates were analysed by western blotting with antibodies against Eg2 and TPX2. **b**, Exogenously added importin-α/β inhibit TPX2-stimulated phosphorylation of Eg2. Bacterially expressed Eg2–His was added into CSF egg extracts supplemented with buffer, or TPX2 with or without exogenous importin-α/β, Ran^{L43E}, Ran^{Q69L} or Ran^{T24N}. Eg2–His was re-isolated using nickel–agarose beads and analysed by western blotting. The same experiments were also performed with endogenous Eg2 using the 1C1 antibody. **c**, Ran and importin-α/β regulate phosphorylation of Eg2 through TPX2 and microtubules *in vitro*. Baculovirus-expressed Eg2 was incubated with microtubules and/or TPX2 with or without importin-α/β, Ran^{L43E}, Ran ^{Q69} or Ran^{T24N}. The phosphorylation status of Eg2 was analysed by western blotting.

expressed Eg2. Phosphorylation of bacterially expressed Eg2 is probably caused by Eg2 itself, because $Eg2^{K169R}$ is not phosphorylated.

As TPX2 and microtubules have no effect on the kinase activity of bacterially expressed Eg2, we wondered why TPX2 and microtubules could activate Eg2 from baculovirus and in *Xenopus* egg extracts. Previous studies suggested that activation of Eg2 by phosphorylation is negatively regulated by PPI (refs 9, 11–13). One possibility is that TPX2 and microtubules are only required to counteract PPI to keep the kinase in the activated form. As PPI is not expressed in *E. coli*, bacterially expressed Eg2 does not require TPX2 and microtubules to exist in a phosphorylated state. If so, introducing PPI into bacterially expressed Eg2 would make phosphorylation of Eg2 dependent on TPX2 and microtubules. We treated the bacterially expressed Eg2 (phosphorylated) with purified PPI and found that phosphorylation of Eg2 on Thr 295 was lost; however, addition of TPX2 and microtubules induced the rephosphorylation of Eg2 in the presence of PPI (Fig. 2e). Therefore, TPX2 and microtubules activate phosphorylation of Eg2 by counteracting PPI.

To determine whether TPX2 and microtubules activate Eg2 by counteracting PPI in the egg extracts, we added bacterially expressed (phosphorylated) histidine-tagged Eg2 into egg extracts supplemented with XB buffer, okadiac acid (OA), inhibitor 2 (I2, specific for PPI) or TPX2 (with or without nocodazole). We then re-isolated Eg2–His with nickel–agarose beads. Western blotting showed that phosphorylation of bacterially expressed Eg2 on Thr 295 was greatly reduced when incubated in the egg extract (Fig. 2f). However, treatment with OA, I2 or TPX2 (in the absence of nocodazole) prevented dephosphorylation (Fig. 2f). As Aurora A is known to interact with PPI (ref. 11), we suspected that the baculovirus-expressed Eg2 was also kept inactive by the copurifying PPI. To test this, we treated purified baculovirus-expressed Eg2 with OA, I2, or TPX2 and microtubules, and found that phosphorylation of Eg2 on Thr 295 was activated in all cases (Fig. 2g). Therefore, TPX2 and microtubules activate both Eg2 from egg extracts and baculovirus-expressed Eg2 by counteracting PPI.

We showed above that Ran-GTP enhances the interaction between TPX2 and Eg2 in the CSF egg extracts (Fig. 1). As importin-α/β was hypothesized to inhibit TPX2 function[30], we asked whether importin-α/β could inhibit the Ran-GTP-stimulated interaction between TPX2 and Eg2. The egg extract was treated with Ran^{L43E} or Ran^{Q69L} (another commonly used activated allele of Ran[30]) with or without addition of exogenous importin-α/β. Eg2 was then immunoprecipitated with the 1C1 antibody and analysed by western blotting. We found that addition of exogenous importin-α/β inhibited the interaction between TPX2 and Eg2 that was stimulated by Ran^{L43E} or Ran^{Q69L} (Fig. 3a).

To determine whether phosphorylation of Eg2 was also inhibited by importin-α/β in egg extracts, we added the bacterially expressed Eg2–His into egg extract supplemented with combinations of TPX2, Ran^{L43E}, Ran^{Q69L}, Ran^{T24N} and importin-α/β. Eg2–His was re-isolated from the egg extract using nickel–agarose beads. Similar experiments were also performed with endogenous Eg2 through immunoprecipitation with the 1C1 antibody. We found that addition of exogenous importin-α/β markedly inhibited TPX2-induced phosphorylation of Eg2 (Fig. 3b). Furthermore, addition of Ran^{L43E} or Ran^{Q69L} alleviated this inhibition. Therefore, one consequence of Ran-GTP-stimulated release of TPX2 from importin-α/β is to stimulate activation of Eg2.

Next, we sought to reconstitute the regulation of Eg2 phosphorylation by Ran-GTP and importin-α/β *in vitro* with purified proteins. Baculovirus-expressed Eg2 (un-phosphorylated) was incubated with different combinations of TPX2, microtubules, importin-α/β, Ran^{L43E}, Ran^{Q69L} and Ran^{T24N}, before analysis by western blotting. Importin-α/β completely inhibited phosphorylation of Eg2 induced by TPX2 and microtubules, whereas Ran^{L43E} and Ran^{Q69L} greatly alleviated this inhibition (Fig. 3c). Therefore, activation of Eg2 by TPX2 and microtubules is regulated by Ran-GTP and importin-α/β.

To determine whether Ran-GTP directly stimulates phosphorylation of Eg2 in a TPX2-dependent manner in egg extracts, we added Ran^{L43E} or Ran^{Q69L} into extracts depleted of TPX2, mock-depleted extract, or TPX2-depleted extract to which bacterially produced TPX2 had been added back (Fig. 4a). Immunoprecipitation and western blotting showed that both Ran alleles stimulated phosphorylation of Eg2 on Thr 295 in the presence of TPX2, but failed to do so in TPX2-depleted egg extract (Fig. 4b). Therefore, phosphorylation of Eg2 at Thr 295 requires both Ran-GTP and TPX2 in *Xenopus* egg extracts.

To determine whether Eg2 is autophosphorylated in egg extract in response to stimulation with Ran-TPX2, we added bacterially

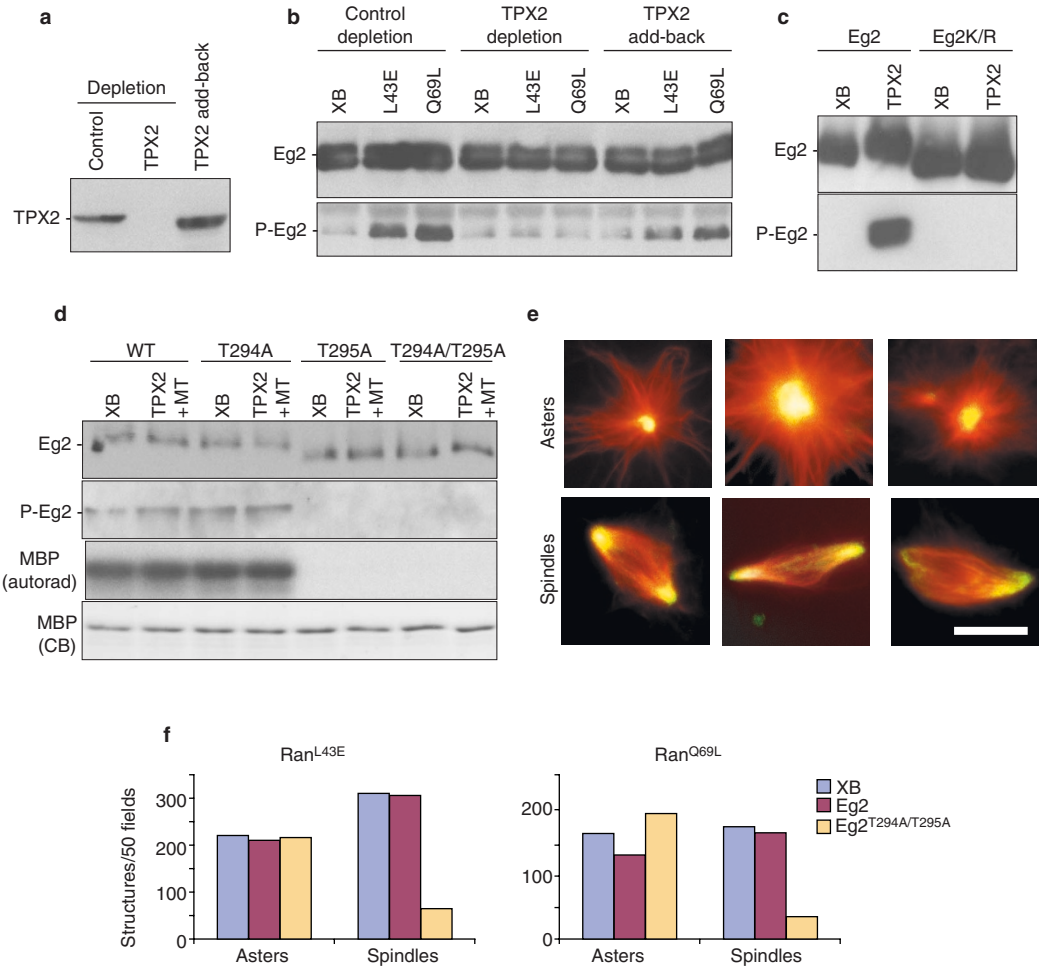

Figure 4 **Phosphorylation of Eg2 is essential for spindle assembly. a**, Egg extracts were depleted with the TPX2 antibody or mock-depleted with control normal rabbit IgG. Purified TPX2 was added back into the egg extract depleted of TPX2. **b**, Ran-GTP-stimulated phosphorylation of Eg2 requires TPX2. Ran^{L43E} or Ran^{Q69L} were added into egg extracts treated as in **a**. Eg2 was immunoprecipitated and analysed by western blotting. **c**, TPX2 stimulates autophosphorylation of Eg2 in egg extracts. Purified His-tagged Eg2 or Eg2K/R were added to egg extracts in the presence or absence of exogenous TPX2. Nickel–agarose beads were used to pull down Eg2. The phosphorylation states of Eg2 were analysed by western blotting with antibodies against either Eg2 or Eg2 phosphorylated on Thr 295. **d**, Phosphorylation of Thr 295 is essential for activation of Eg2. Wild-type Eg2, $Eg2^{T294A}$, $Eg2^{T295A}$ or $Eg2^{T294A/T295A}$ were incubated with XB buffer or TPX2 and microtubules. They were then used to phosphorylate MBP before separation by SDS–PAGE and analysis by Coomassie Blue staining (CB) and autoradiography (autorad). Eg2 was detected by western blotting with antibodies against Eg2 or Eg2 phosphorylated at Thr 295. **e**, Immunofluorescence microscopy images of three typical microtubule asters and spindles. Microtubule asters and spindles were labelled with rhodamine–tubulin (red) and immunostained with anti-γ-tubulin (green) to mark the polarity of the structures. Colocalization of γ-tubulin and microtubules is shown in yellow. Scale bar represents 10 μm. **f**, The number of asters and spindles in **e** were counted from 50 random fields. $Eg2^{T294A/T295A}$ disrupts Ran-induced bipolar spindle assembly.

expressed wild-type Eg2–His or $Eg2^{K169R}$–His and stimulated the extracts with TPX2. Phosphorylation of Eg2–His was then analysed by western blotting. We found that wild-type Eg2–His became phosphorylated at Thr 295 after TPX2 stimulation, whereas $Eg2^{K169R}$–His did not (Fig. 4c). This suggests that the Ran–TPX2 pathway stimulates auto-phosphorylation of Eg2 in the egg extract.

We have shown that phosphorylation of Eg2 on Thr 295 is essential for its kinase activity and that the Ran–TPX2 pathway is essential for this phosphorylation in egg extracts. If phosphorylation of Thr 295 is essential for Eg2 function in spindle assembly, unphosphorylatable Eg2 might inhibit spindle assembly. There are two threonines in the activation loop of Eg2 (Thr 294 and Thr 295). We mutated either or both of the threonines to alanines to create $Eg2^{T294A}$, $Eg2^{T295A}$ and $Eg2^{T294A/T295A}$. We purified wild-type and mutant Eg2 and assayed their kinase activity. As expected, Eg2 and $Eg2^{T294A}$, but not $Eg2^{T295A}$ and $Eg2^{T294A/T295A}$, were phosphorylated on Thr 295 and had similar kinase activities toward MBP (Fig. 4d). Consistent with our earlier observations, TPX2 and microtubules had no effect on the kinase activity of these bacterially expressed enzymes (Fig. 4d).

To determine whether phosphorylation of Eg2 Thr 295 is essential for Ran-GTP-stimulated spindle assembly, we added wild-type Eg2, $Eg2^{T295A}$ and $Eg2^{T294A/T295A}$ to the egg extracts at levels approximately fourfold in excess of endogenous Eg2. We found that both $Eg2^{T295A}$ and $Eg2^{T294A/T295A}$, but not wild-type Eg2, greatly reduced the number of spindles stimulated by Ran. However, microtubule aster assembly was unaffected. Figure 4e shows three typical spindles and asters that are quantified in Fig. 4f. As $Eg2^{T295A}$ and $Eg2^{T294A/T295A}$ exhibited similar inhibitory effects, only the data for $Eg2^{T294A/T295A}$ are shown (Fig. 4f). Interestingly, assembly of microtubule asters still occurred in the presence of mutant Eg2, suggesting that the Ran pathway mediated by Eg2 does not significantly influence

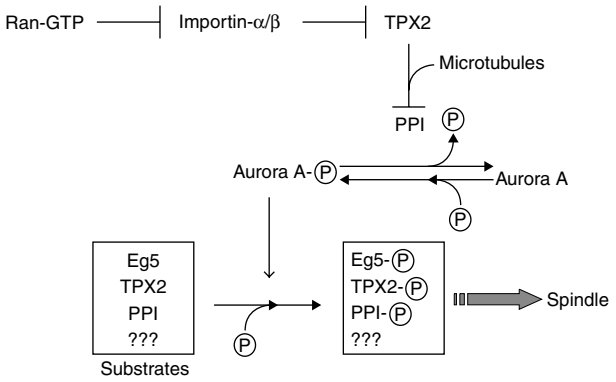

Figure 5 **A pathway for Ran-stimulated assembly.** We propose that one pathway in Ran-stimulated spindle assembly targets Aurora A kinase. Ran-GTP relieves the inhibitory binding of importin-α/β on TPX2. This allows TPX2 and microtubules to block PPI from inactivating Aurora A. Active Aurora A could then phosphorylate its substrates, including Eg5 (ref. 35), TPX2 (ref. 34) and PPI (ref. 11), which could contribute to spindle assembly.

microtubule aster assembly in mitosis.

Taken together, these observations show that phosphorylation on Thr 295 is essential for Eg2 function in spindle assembly. As phosphorylation of Thr 295 is completely dependent on the Ran–TPX2 pathway, we conclude that one function of Ran-GTP in spindle assembly is to activate Eg2 phosphorylation through TPX2 and microtubules. Our findings identify a novel Ran-GTP-mediated signalling pathway involved in mitosis. Ran-GTP stimulates spindle assembly by relieving the inhibitory binding of importin-α/β on TPX2, which in turn counteracts PPI to activate Eg2 (Fig. 5). We further hypothesize that Ran-stimulated activation of Eg2 could result in phosphorylation of multiple substrates, including Eg5 (ref. 35), which in turn establishes and/or maintains the bipolar spindle (Fig. 5).

Ran is a member of the low-molecular-weight GTPase family that includes Ras, Rho, Rab and Arf. Although many small GTPases bind directly to diverse downstream targets, including kinases to transduce signals, Ran was thought to regulate the assembly and disassembly of protein complexes through binding to a family of nuclear transport receptors that contain a conserved Ran-binding domain. Our findings demonstrate that Ran, in common with other small GTPases, also transmits signals through a protein kinase, albeit by a different mechanism. It will be interesting to investigate whether Ran regulates additional mitotic kinases.

The microtubule-dependent activation of Eg2 demonstrated in this study is particularly intriguing because it provides a mechanistic basis for the orderly assembly of the mitotic spindle. We suggest that the microtubule-dependent Eg2 activation provides an efficient mechanism to interpret the Ran-GTP gradient established by the chromosomes during spindle assembly. In a normal cell, activation of Eg2 could be initiated on a few microtubules assembled near the condensed chromosomes, where the Ran-GTP concentration is high. The initial activation could stimulate additional microtubule nucleation[6] and organization, which in turn would amplify Eg2 activity. Therefore, the Eg2 feedback amplification loop dictated by the Ran-GTP concentration would ensure that microtubule assembly and organization occur in a coordinated manner. It is also interesting to note that although TPX2 and Eg2 are concentrated at the spindle poles (which may be caused by the high density of microtubules at the poles), both TPX2 and Eg2 are also found on the microtubules near the spindle mid-zone, where the Ran-GTP concentration is high[25,36]. The Ran-GTP gradient established by the chromosomes could be translated into the Eg2 kinase gradient on the microtubules to regulate spindle assembly.

Our findings show that Ran-GTP regulates the balance between the Aurora A kinase (Eg2) and PPI in spindle assembly. PPI may continuously inactivate Aurora A, creating generally unfavourable conditions for spindle assembly. Alternatively, Ran-GTP generated by the condensed chromosomes would locally override the activity of PPI through TPX2 and microtubules, therefore activating Aurora A and spindle assembly on the existing microtubules. Spindle assembly, similar to many other processes in mitosis, is regulated by phosphorylation and dephosphorylation. By regulating the balance between Aurora A and PPI, the Ran signalling pathway may control a subset of the phosphorylation and dephosphorylation events during spindle formation.

Deregulation of Aurora A kinase has been implicated in tumorigenesis[37–39]. Several studies have shown that overexpression of Aurora A kinase causes aneuploidy in tissue culture cells. In addition, several tumour types were found to have elevated levels of the Aurora family of kinases, including Aurora A. As both Aurora A kinase and TPX2 are components of a signalling pathway regulated by Ran in spindle assembly, our findings provide a plausible cause of tumorigenesis. Overexpression of Aurora A or TPX2 may result in constitutive activation of Aurora A kinase, which could then uncouple the Ran signalling pathway. This uncoupling could cause uncoordinated spindle assembly and defects in chromosome segregation. □

Methods

Production and purification of recombinant proteins

His(6×)-tagged importin- α/β, Ran^{L43E} and Ran^{Q69L} were purified using nickel–agarose beads, as described[30–32]. These proteins were concentrated to 5–20 mg ml^{-1}, snap-frozen in small aliquots in liquid nitrogen and stored at −80 °C. Samples were used at 0.5–1 mg ml^{-1} final concentration in all assays. GST–TPX2 was constructed by fusing TPX2 in-frame with GST in pGEX-6p-2. The fusion protein was expressed in *E. coli* strain BL21DE3 before purification with GST–agarose beads. Cleaved TPX2 was prepared by treating GST–TPX2 with Precision Protease (Amersham, New York, NY). GST–TPX2 and cleaved TPX2 were concentrated to 5–10 mg ml^{-1}, stored at −80 °C and used at 0.5–1 μM. Bacterially expressed wild-type Eg2–His and mutant Eg2–His ($Eg2^{K169R}$, $Eg2^{T294A}$, $Eg2^{T295A}$ and $Eg2^{T294A/T295A}$) or baculovirus-expressed wild-type Eg2 were purified in the absence of phosphatase inhibitors, as described[7,8,13], and stored frozen at 5–10 mg ml^{-1}. All proteins were stored in XB buffer[33]. The Eg2 point mutation was created with mutagenizing primers using a bacterial construct expressing wild-type Eg2–His[7,8]. Mutations were confirmed by sequencing. The endogenous concentration of Eg2 was estimated to be ~0.8 μM by western blotting using purified Eg2 as a standard.

Production and purification of antibodies

The 1C1 monoclonal antibody has been previously described[7,8]. The polyclonal antibodies 966 and 1807 were raised against the carboxy (PKKKDEPLAGAQ)- and amino (MTEGPKRIPVSQC)-terminal peptides of Eg2. The polyclonal antibody against full-length Eg2 was raised against the bacterially expressed Eg2 and affinity purified. This antibody was used to detect Eg2 on western blots. The polyclonal antibody against phospho-Thr 295 of Eg2 was raised against a phosho-peptide, (C)APSSRRT-pT-L, before purification first against the non-phosphorylated peptide, (C)APSSRRTTL, and then the phosphorylated peptide. The polyclonal antibody against TPX2 was raised against GST–TPX2 before purification against TPX2.

Egg extract preparation, immunoprecipitation and kinase assays

The CSF egg extract was prepared as described[33]. One round of TPX2 immunoprecipitation using 80–100 μg affinity purified TPX2 antibody resulted in an 80–90% reduction of TPX2 in 100 μl of extract. Eg2 was immunoprecipitated from the CSF egg extracts using 1C1, 966 or 1807 antibodies and the immunoprecipitates were washed with XB buffer. The immunoprecipitation and washes were performed at room temperature. Immunoprecipitates were either analysed by western blotting to determine the phosphorylation state of Eg2 or used for kinase assays. For the kinase assay, Eg2 was incubated with 0.25 μCi γ-^{32}P-ATP with or without MBP (0.25 mg ml^{-1} final concentration) at room temperature for 5 min. After separation by SDS–PAGE, phosphorylated MBP was detected by autoradiography. Nocodazole (100 μM; Sigma, St Louis, MO) was used to inhibit microtubule assembly in egg extracts.

Phosphatase assays

PPI (Upstate, Charlottesville, VA) was used at 5U ml^{-1} (final concentration) to treat purified bacterially expressed Eg2 (1 μM) for 20 min at room temperature. TPX2 (0.5 μM) and taxol-stabilized microtubules (1 mg ml^{-1} tubulin) were added and incubated for additional 10 min at room temperature before separation by SDS–PAGE and analysis by western blotting. For the assays in which phosphatase inhibitors were used, the same amount of proteins as above were used in the presence of 3 μM OA (Sigma) and 2 μM I2 (New England Biolabs, Beverly. MA).

Spindle assembly assays

Spindle assembly was induced using Ran^{L43E} or Ran^{Q69L} (refs 23, 31). Purified wild-type Eg2 or mutant $Eg2^{T295A}$ and $Eg2^{T294A/T295A}$ were added to the spindle assembly reactions (~3.2 μM final concentration). Spindles were spun onto coverslips before immunostaining with an anti-γ-tubulin antibody (Sigma) and analysis[23,31]. Experiments were repeated four times with four different egg extracts. Similar results were obtained in all cases.

RECEIVED 13 NOVEMBER 2002; REVISED 16 JANUARY 2003; ACCEPTED 16 JANUARY 2003; PUBLISHED 10 FEBRUARY 2003.

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ACKNOWLEDGEMENTS

We thank S. Lizarraga for help with egg extracts, M. Inskeep for technical support, D. Golick for His-tagged Ran^{Q69L}, E. Lees for the anti-Eg2 antibody, M. Guo and the members of the Zheng lab for comments. Supported by the Howard Hughes Medical Institute (Y.Z.), by CNRS, ARC, and LNCC of France (C.P.) and by National Institutes of Health grant HD23696 (J.R.).

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.