Canoe binds RanGTP to promote Pins\textsuperscript{TPR}/Mud-mediated spindle orientation

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Regulated spindle orientation maintains epithelial tissue integrity and stem cell asymmetric cell division. In Drosophila melanogaster neural stem cells (neuroblasts), the scaffolding protein Canoe (Afadin/AF-6 in mammals) regulates spindle orientation, but its protein interaction partners and mechanism of action are unknown. In this paper, we use our recently developed induced cell polarity system to dissect the molecular mechanism of Canoe-mediated spindle orientation. We show that a previously uncharacterized portion of Canoe directly binds the Partner of Inscuteable (Pins) tetratricopeptide repeat (TPR) domain. The Canoe–Pins\textsuperscript{TPR} interaction recruits Canoe to the cell cortex and is required for activation of the Pins\textsuperscript{TPR}/Mud (nuclear mitotic apparatus in mammals) spindle orientation pathway. We show that the Canoe Ras-association (RA) domains directly bind RanGTP and that both the Canoe\textsuperscript{RA} domains and RanGTP are required to recruit Mud to the cortex and activate the Pins/Mud/dynein spindle orientation pathway.

Introduction

Spindle orientation is essential to maintain epithelial integrity; planar spindle orientation results in both daughter cells maintaining apical junctions and remaining part of the epithelium, whereas apical/basal spindle orientation can lead to the loss of the basal daughter cell from the epithelium (Lu et al., 2001; Egger et al., 2007). Spindle orientation is also important during asymmetric cell division of stem, progenitor, and embryonic cells; when the spindle orients along an axis of intrinsic or extrinsic polarity, it will generate two distinct daughter cells, but, when the spindle aligns perpendicular to the axis of polarity, it will generate two identical daughter cells (Cabernard and Doe, 2009; Siller and Doe, 2009). Proper spindle orientation may even be necessary to prevent tumorigenesis (Gonzalez, 2007; Fleming et al., 2009; Quyn et al., 2010). Thus, it is essential to understand the molecular mechanisms that regulate spindle orientation, particularly those that use evolutionarily conserved proteins and pathways, to help direct stem cell lineages and potentially treat pathological conditions caused by aberrant spindle orientation.

Drosophila melanogaster neural stem cells (neuroblasts) provide an excellent system for studying spindle orientation during asymmetric cell division. Neuroblasts have an apical/basal polarity and orient their mitotic spindle along this cortical polarity axis to generate distinct apical and basal daughter cells. The apical neuroblast inherits fate determinants responsible for neuroblast self-renewal, whereas the basal daughter cell inherits fate determinants responsible for neuronal/glial differentiation (Doe, 2008). Genetic studies have identified proteins that regulate spindle orientation during asymmetric cell division, including the apically localized proteins Inscuteable, Partner of Inscuteable (Pins; LGN/AGS-3 in mammals), Mushroom body defect (Mud; nuclear mitotic apparatus [NuMA] in mammals), Discs large (Dlg), and G\textalpha_i (Doe, 2008). In addition, many proteins that are not asymmetrically localized are required for spindle orientation, including the dynein complex and the Aurora A and Polo kinases (Siller and Doe, 2009).

We have recently developed an induced cell polarity/spindle orientation system using the normally apolar S2 cell line to biochemically dissect Drosophila and vertebrate spindle orientation (Johnston et al., 2009; Ségalen et al., 2010). Using this system to characterize Drosophila spindle orientation, we showed that cortical Pins nucleates two spindle orientation pathways: (1) the Pins\textsuperscript{LINKER} domain is phosphorylated by regulated spindle orientation maintains epithelial tissue integrity and stem cell asymmetric cell division. In Drosophila melanogaster neural stem cells (neuroblasts), the scaffolding protein Canoe (Afadin/AF-6 in mammals) regulates spindle orientation, but its protein interaction partners and mechanism of action are unknown. In this paper, we use our recently developed induced cell polarity system to dissect the molecular mechanism of Canoe-mediated spindle orientation. We show that a previously uncharacterized portion of Canoe directly binds the Partner of Inscuteable (Pins) tetratricopeptide repeat (TPR) domain. The Canoe–Pins\textsuperscript{TPR} interaction recruits Canoe to the cell cortex and is required for activation of the Pins\textsuperscript{TPR}/Mud (nuclear mitotic apparatus in mammals) spindle orientation pathway. We show that the Canoe Ras-association (RA) domains directly bind RanGTP and that both the Canoe\textsuperscript{RA} domains and RanGTP are required to recruit Mud to the cortex and activate the Pins/Mud/dynein spindle orientation pathway.

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The PinsTPR domain binds the CanoePBD domain

Next, we mapped the Canoe domain that interacts with the PinsTPR. We expressed in S2 cells a series of N-terminal truncations of Canoe, which all targeted to the cortex as a result of the C-terminal actin-binding domain. We found that all of the known Canoe domains (RA, Forkhead, Dilute, and PDZ; Fig. 2 A) were dispensable for Canoe-PinsTPR association (Fig. 2 B–I). Next, we made C-terminal deletions to define the C-terminal Canoe domain necessary and sufficient for recruiting PinsTPR to the cortex. Because C-terminal deletions of Canoe lack the actin-binding domain necessary for cortical localization, we tethered Canoe to the cortex by fusing it in frame to the C terminus of the transmembrane Echinoid (Ed) protein, which is an established method for obtaining cortical localization of proteins and protein domains (Johnston et al., 2009; Ségalan et al., 2010). We found that a Canoe protein that contained just the 1,755–1,950 domain effectively recruited PinsTPR to the cortex, as did larger fragments that contained this domain, whereas proteins lacking this domain failed to recruit PinsTPR to the cortex (Fig. 2, J–M). We term this domain the PinsTPR domain (PBD; shown as a black box in Fig. 2 A). We conclude that the CanoePBD is necessary and sufficient for Canoe-PinsTPR cortical association.

To test whether the CanoePBD–PinsTPR interaction is direct, we generated GST:Canoe fusions and assayed for PinsTPR binding using purified proteins in pull-down assays. GST alone or GST:Canoe proteins lacking the PBD failed to bind PinsTPR (Fig. 2 N). When expressed alone, GFP-tagged full-length Canoe protein localized uniformly to the cell cortex of S2 cells (Fig. 1 C), whereas a Cherry-tagged full-length Pins localized evenly through the cytoplasm (Fig. 1 D). We reasoned that if there is an interaction between Canoe and Pins, coexpression of the two proteins should result in recruitment of Pins to the cell cortex. Indeed, coexpression of the full-length Pins and Canoe proteins resulted in Pins recruitment to the cell cortex (Fig. 1 E, arrowhead). Next, we coexpressed full-length Canoe with different Pins domains and assayed for Pins cortical recruitment. We found that only the PinsTPR domain was sufficient to recruit Pins to the cortical Canoe domain (Fig. 1, F–H). We conclude that the PinsTPR domain is necessary and sufficient for Pins-Canoe cortical colocalization.

Results and discussion

The PinsTPR domain is necessary and sufficient for Pins-Canoe cortical colocalization

Canoe and Pins are colocalized at the cortex of mitotic neuroblasts (Speicher et al., 2008). In this section, we test which Pins domain is necessary and sufficient to recruit Canoe to the cortex in S2 cells (Canoe and Pins protein domains shown in Fig. 1, A and B); in the next section, we test which Canoe domain is required to recruit Pins to the cortex and test each domain for direct binding using in vitro pull-down assays.

by Aurora A, which allows recruitment of Dlg, which interacts with the kinesin Khc-73 to promote partial spindle orientation; and (2) the Pins tetrapartitepeptide repeat (TPR) domain (PinsTPR) binds Mud, which promotes dynein–dynactin complex-mediated spindle orientation (Johnston et al., 2009). We also used this induced cell polarity system to characterize Dishevelled-mediated spindle orientation in the zebrafish embryo and in Drosophila sensory organ precursor cells, identifying a Dishevelled domain that is necessary and sufficient to bind Mud and regulate spindle orientation in both cell types (Ségalan et al., 2010).

The scaffolding protein Canoe has been shown to regulate spindle orientation and cell polarity in Drosophila neuroblasts (Speicher et al., 2008), although the mechanisms involved remain unknown. Canoe contains two Ras-association (RA) domains, a Forkhead domain, a myosinlike Dilute domain, and a PSD-95, Dlg, and ZO-1 (PDZ) domain. In addition to regulating neuroblast cell polarity and spindle orientation, it integrates Notch, Ras, and Wnt pathways during Drosophila muscle progenitor specification (Carmena et al., 2006) and serves as a Rap1 effector within the Jun N-terminal kinase pathway during dorsal closure of the Drosophila embryo (Takahashi et al., 1998; Boettner et al., 2003), and the mammalian orthologue Afadin links cadherins to the actin cytoskeleton at adherens junctions (Mandai et al., 1997; Sawyer et al., 2009). Here, we map direct Pins/Canoe and Canoe/RanGTP-binding domains and use the induced cell polarity/spindle orientation system to show that Canoe/RanGTP is required for Pins to recruit Mud and activate the Pins/Mud/dynein spindle orientation pathway.

Figure 1. PinsTPR and Canoe colocalize in interphase S2 cells.

(A and B) Domain structure of Canoe and Pins. Dil, Dilute; FHA, Forkhead. (C and D) Single protein expression in S2 cells. GFP-tagged Canoe binds to the cortex (C), whereas Cherry-tagged full-length Pins (Pins) is not cortical (D). (E–H) Coexpression of Canoe and Pins in S2 cells. GFP:Canoe recruits Cherry:Pins proteins containing the TPR domain (PinsTPR, PinsTPR+LINKER, and PinsTPR+LINKER+GL) to the cortex (arrowheads) but not a protein lacking the TPR domain. GL, GoLoco domain. Bar, 5 µm.
molecule in S2 cells results in clustering of the Ed protein to the site of cell contact as a result of homophilic adhesion of the extracellular Ed domain, creating a polarized distribution of Ed at the cell cortex. Fusion of any test protein or protein domain to the C terminus of Ed allows us to create a cortical crescent of the test protein and assay for its function in spindle orientation during mitosis. For example, Ed:PinsTPR+LINKER gives excellent spindle orientation of <15°; Ed:Pins LINKER only gives partial spindle orientation of 30° as a result of the absence of the TPR part of the pathway, and the Ed:GFP control gives random spindle orientation of 45° (Johnston et al., 2009).

Here, we use this assay to test the role of Canoe in Pins-mediated spindle orientation. We confirm that Ed:GFP alone had no spindle orientation activity (49 ± 30°; quantified in Fig. 3 H; Fig. 3 A), whereas Ed:PinsTPR+LINKER showed excellent spindle orientation of 13 ± 8° as a result of the absence of the Canoe PBD alone (Fig. 2 N). We conclude that the Canoe PBD directly binds the Pins TPR domain and suggest that this interaction is responsible for the cortical Pins–Canoe interaction in S2 cells (Figs. 1 and 2) and mitotic neuroblasts (Speicher et al., 2008).

**Canoe is required for PinsTPR/Mud-mediated spindle orientation**

Based on the observed binding of Canoe to the PinsTPR domain, we next tested whether Canoe is part of the PinsTPR/Mud/dynein spindle orientation pathway. To assay spindle orientation in S2 cells, we need to create a localized cortical domain of protein so we can determine whether the spindle aligns with this domain. To do this, we used our recently developed induced cell polarity/spindle orientation assay (Johnston et al., 2009). In this assay, expression of the Ed transmembrane cell adhesion molecule in S2 cells results in clustering of the Ed protein to the site of cell contact as a result of homophilic adhesion of the extracellular Ed domain, creating a polarized distribution of Ed at the cell cortex. Fusion of any test protein or protein domain to the C terminus of Ed allows us to create a cortical crescent of the test protein and assay for its function in spindle orientation during mitosis. For example, Ed:PinsTPR+LINKER gives excellent spindle orientation of <15°; Ed:PinsLINKER only gives partial spindle orientation of ~30° as a result of the absence of the TPR part of the pathway, and the Ed:GFP control gives random spindle orientation of ~45° (Johnston et al., 2009).

Here, we use this assay to test the role of Canoe in Pins-mediated spindle orientation. We confirm that Ed:GFP alone had no spindle orientation activity (49 ± 30°; quantified in Fig. 3 H; Fig. 3 A), whereas Ed:PinsTPR+LINKER showed excellent spindle orientation (13 ± 8°; quantified in Fig. 3 H; Fig. 3 B). RNAi knockdown of endogenous Canoe in S2 cells

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**Figure 2.** Canoe1755-1950 is necessary and sufficient for PinsTPR binding. (A) Domain architecture of full-length Canoe protein. Amino acids are shown in gray. Dil, Dilute; FHA, Forkhead. (B–I) Coexpression of Canoe and Pins in S2 cells. All tested GFP:Canoe N-terminal–truncated proteins (numbers represent amino acids present in protein) recruit Cherry:PinsTPR to the cortex. [J–M] Coexpression of Ed:GFP:Canoe domains with Cherry:PinsTPR in S2 cells. Only Canoe proteins containing the 1,755–1,950 domain recruited PinsTPR to the cortex (arrowheads). (N) Canoe1755–1950 directly binds the PinsTPR domain. GST:Canoe fragments were incubated with His-tagged PinsTPR protein and probed for Canoe-dependent binding of PinsTPR. (top) Coomassie stain of purified GST:Canoe protein fragments or His-tagged PinsTPR protein (rightmost lane). (bottom) Western blot to detect bound PinsTPR. Bars, 5 µm.
expressing Ed:GFP resulted in partial spindle orientation (27 ± 21°; quantified in Fig. 3 H; Fig. 3, C and D), as expected for a functional Pins LINKER pathway in the absence of the Pins TPR/Mud/dynein pathway (Johnston et al., 2009). Canoe RNAi reduced endogenous protein levels (Fig. S2) and did not result in mitotic spindle abnormalities (Fig. S1), suggesting that the spindle orientation phenotypes were not caused by a decrease in astral microtubules or spindle microtubules. Importantly, double RNAi knockdown of canoe and mud together did not significantly enhance the canoe single RNAi phenotype (33 ± 25°; quantified in Fig. 3 H; Fig. 3 E), consistent with both proteins acting in the same pathway. We conclude that Canoe is part of the Pins TPR/Mud/dynein spindle orientation pathway.

**Canoe RA domains are required for spindle orientation**

To assess what protein domains of Canoe are necessary and sufficient for it to stimulate Pins/Mud-mediated spindle orientation, we performed spindle orientation rescue assays with Canoe deletion constructs. In this assay, Ed:Pins TPR+LINKER and a Canoe deletion allele were coexpressed in S2 cells while endogenous Canoe levels were reduced using RNAi targeted to the canoe 3′ untranslated region (UTR). The canoe 3′ UTR RNAi probe reduced Pins-mediated spindle orientation similar to the canoe coding sequence RNAi probe (32 ± 22°; quantified in Fig. 3 H; Fig. 3, C and D).

We then coexpressed Ed:Pins TPR+LINKER with Canoe deletion alleles in a canoe 3′ UTR RNAi background. We reasoned that the truncation of the necessary domains responsible for mediating spindle orientation would fail to rescue spindle orientation. Interestingly, deletion of both RA domains failed to rescue Pins-mediated spindle orientation (32 ± 24°; quantified in Fig. 3 H; Fig. 3 G). We conclude that the Canoe RA domains are required for spindle orientation.

**Figure 3. Canoe regulates Pins-mediated spindle orientation.** (A–G) S2 cell spindle orientation assay. Representative images are shown, and the quantification of each experiment is shown in H. Ed:GFP or Ed:GFP:OII proteins were induced to form cortical crescents by cell aggregation, and the angle of the mitotic spindle was measured relative to the center of the cortical crescent. Pins cortical localization (green), mitotic spindle [α-tubulin [αTub]], and merge (in some cases also showing the mitotic DNA marker phospho–histone H3 [PH3]) are shown. CDS, coding sequence. Bar, 5 µm. (H) Quantification of experiments shown in A–G depicted as a cumulative plot. Random spindle orientation is a diagonal line (e.g., Ed:GFP); optimal spindle orientation is reflected in a leftward shift in the plot (e.g., Ed:Pins TPR+LINKER), and partial spindle orientation falls in between. The key is an abbreviated version of the experiments shown on the left in A–G. Cno CDS RNAi, n = 36; Cno 3′ UTR RNAi, n = 36; CnoFL Rescue, n = 30; Cno Delta RA Rescue, n = 30; Cno RNAi + Mud RNAi, n = 29; Ed:Pins TPR+LINKER, n = 30; Ed:GFP, n = 33.
RanGTP binds CanoeRA domains and is required for Pins\textsuperscript{TPR}/Mud-mediated spindle orientation

RA domains are known to bind small monomeric GTPases such as Ran, Ras, and Rap1 (Kuriyama et al., 1996; Boettner et al., 2003; Dallol et al., 2009). Because Ran is the small GTPase most closely linked to the mitotic spindle assembly and function (Kalab and Heald, 2008), we tested whether Ran binds Canoe RA domains and, if so, whether it regulates spindle orientation. We made GST fusions with full-length Canoe or the individual RA1 and RA2 domains and tested whether they could interact with purified Ran loaded with the GTP analogue GMPPNP or GDP. We found that RanGTP preferentially bound Canoe full-length (Canoe\textsuperscript{FL}), RA1, or RA2 proteins (Fig. 4 A, third, fifth, and seventh lanes) compared with RanGDP (Fig. 4 A, fourth, sixth, and eighth lanes). The negative control GST alone did not bind appreciable RanGTP nor RanGDP (Fig. 4, first and second lanes). Furthermore, Ran communoprecipitated with Canoe from S2 cells and also localized to Ed:CanoeRA crescents (Fig. 4 B–D). We conclude that the Canoe RA domains can interact directly with GTP-loaded Ran.

Next, we asked whether Ran is necessary for Pins\textsuperscript{TPR}/Mud-mediated spindle orientation. To address this question, we performed RNAi knockdown of endogenous Ran in S2 cells expressing Ed:Pins\textsuperscript{TPR+LINKER} and found that spindle orientation was reduced to 34 ± 27° (quantified in Fig. 4 I; Fig. 4 E). ran RNAi reduced endogenous protein levels without affecting centrosome number or spindle morphology (Figs. S1 and S2). The effect of ran RNAi on spindle orientation is similar to the canoe RNAi phenotype as well as to the amount of spindle orientation.
provided by the Pins\textsuperscript{LINKER} pathway alone after elimination of the Pins\textsuperscript{TPR} pathway components Mud, dynein, or Lis1 (Johnston et al., 2009). To test whether the effects of Ran on spindle orientation are specific to the Pins\textsuperscript{TPR}/Mud pathway, we performed double RNAi knockdowns of \textit{canoe} and \textit{ran} in S2 cells expressing Ed:Pins\textsuperscript{TPR+LINKER} and found that spindle orientation was reduced similar to \textit{canoe} RNAi alone (31 \pm 23\%; quantified in Fig. 4 I; Fig. 4 F), consistent with Ran and Canoe acting in the same pathway. In contrast, double RNAi knockdowns of \textit{ran} and the Pins\textsuperscript{LINKER} pathway component \textit{dlg} led to a more severe spindle orientation phenotype than \textit{ran} RNAi alone (40 \pm 28\%; quantified in Fig. 4 I; Fig. 4 G), consistent with each gene acting in different pathways. To see whether Ran is sufficient to orient the mitotic spindle, we expressed Ed:Ran\textsuperscript{Q69L} (a RanGTP mimic) in S2 cells but were unable to assay its function in spindle orientation because the transmembrane-tethered Ed:Ran protein was trapped in vesicles around the nucleus (Fig. 4 H). We conclude that RanGTP directly binds the Canoe\textsuperscript{RA} domains and is required in a Pins\textsuperscript{TPR}/Canoe/Mud spindle orientation pathway.

**Canoe and RanGTP are required for Mud recruitment to Pins cortical crescents**

How does Canoe/RanGTP promote activity of the Pins\textsuperscript{TPR}/Mud spindle orientation pathway? A prior study showed that \textit{canoe} mutants lack Mud localization to the Pins cortical crescent (Speicher et al., 2008), so we tested whether Canoe is required for Pins/Mud colocalization in our S2 cell assay. We confirm that endogenous Mud is recruited to Ed:Pins\textsuperscript{TPR+LINKER} crescents (Fig. 5 A; Johnston et al., 2009) but that Mud failed to localize with Ed:Pins\textsuperscript{TPR+LINKER} crescents after \textit{canoe} RNAi (Fig. 5 B). Similarly, Mud failed to localize to Ed:Pins\textsuperscript{TPR+LINKER} crescents after \textit{ran} RNAi (Fig. 5 C). To assess whether the Canoe–Ran interaction is necessary for Mud recruitment to Pins crescents, we performed rescue assays with full-length Canoe and RA domain deletion constructs. The full-length Canoe construct rescued endogenous Mud recruitment to Pins crescents, whereas deletion of both RA domains failed to recruit endogenous Mud (Fig. 5, D and E). The requirement for Canoe/RanGTP is specific to the Pins\textsuperscript{TPR} pathway because \textit{canoe} RNAi does not affect endogenous Dlg recruitment to the Pins\textsuperscript{LINKER} (Fig. 5, F–H). We conclude that the Canoe RA domains/RanGTP are required for recruitment of endogenous Mud to cortical Pins\textsuperscript{TPR} crescents and the activation of the Pins\textsuperscript{TPR}/Mud spindle orientation pathway.

How might Canoe/RanGTP promote Mud recruitment to the Pins cortical domain? One model is that Ran sequesters importin-\(\alpha/\beta\) away from the Mud NLS, thereby allowing Mud to interact with Pins. This model is based on the observation that RanGTP inhibits binding of importin-\(\beta\) to the NLS of NuMA (the mammalian orthologue of Mud), increasing the pool of NuMA available to promote spindle formation (Nachury et al., 2001; Wiese et al., 2001). The model predicts that Mud can bind importin-\(\alpha/\beta\) and that this binding prevents Mud/Pins association. Consistent with the model, importin-\(\beta\)/Mud were coimmunoprecipitated from S2 cell lysates (Fig. 5 K), and a GST:Mud fragment containing the adjacent Mud TPR-interacting peptide (TIP)–NLS domains (GST:Mud\textsuperscript{TIP-NLS}) could bind purified importin-\(\beta\) in the presence of importin-\(\alpha\) (Fig. 5 K). However, we found that increasing the concentration of purified importin-\(\alpha/\beta\) did not effect the amount of Pins pulled down with GST:Mud\textsuperscript{TIP-NLS} (Fig. 5 K, first through third and fifth through ninth lanes), which does not support a model in which Ran must sequester importin-\(\alpha/\beta\) to allow Pins/Mud binding. Furthermore, a GFP-tagged Mud\textsuperscript{TIP-NLS} fragment localized to Ed:Pins\textsuperscript{TPR+LINKER} crescents independently of the Canoe/Ran pathway (Fig. 5, I and J), showing that the Mud NLS is not involved in the Canoe/Ran-regulated localization mechanism. Interestingly, Canoe/RanGTP regulation is required for recruitment of full-length endogenous Mud (Fig. 5 B) but not for the recruitment of the smaller Mud\textsuperscript{TIP-NLS} fragment (Fig. 5, I and J); this indicates that Canoe/RanGTP normally functions by blocking an unknown inhibitor of the Mud–Pins\textsuperscript{TPR} interaction.

In conclusion, we have characterized the molecular mechanism by which Canoe regulates spindle orientation. We identified a region of Canoe (amino acids 1,755–1,950) that directly interacted with the Pins\textsuperscript{TPR} domain and showed that these domains are necessary and sufficient for Canoe–Pins association. We showed that the Canoe RA domains bind directly to RanGTP, that both the Canoe RA domains and Ran are necessary for the Pins\textsuperscript{TPR}/Mud spindle orientation pathway, and that Canoe/RanGTP acts by promoting Mud recruitment to the cortical Pins domain. All of the proteins in the Pins/Canoe/Ran/Mud pathway are conserved from flies to mammals, suggesting that this pathway could be widely used to regulate spindle orientation.

**Materials and methods**

**Construction of transgenes and S2 expression**

Echinoid:GFP transgenes were generated within the pMT expression vector as previously described (Johnston et al., 2009; Ségalen et al., 2010); canoe coding sequences were cloned downstream of GFP using 5' Nheli and 3' Not restriction sites. Pins\textsuperscript{TPR+LINKER} and Ran coding sequences were cloned downstream of GFP using 5' BglII and 3' Sall restriction sites. GFP: Canoe, Cherry:Pins, Flag:Ran, HA:importin-\(\beta\), and GFP:Mud were cloned into pMT expression vector alone. <i>Drosophila</i> Schneider (S2) cells were maintained in Schneider's medium with 10% FBS at room temperature. Approximately 3 \(\times\) 10\(^6\) cells were seeded per well in a 6-well plate and transfected with 0.5–0.8 \(\mu\)g total DNA per well using the Effectene manufacturer's protocol and incubated overnight, and gene expression was induced by adding 0.5 mM CuSO\(_4\) for 24 h. Cells were then collected, resuspended in fresh media, and placed in a 6-well plate, and cell clustering was induced by shaking at \(-175\) rpm for 2–3 h.

**RNAi design and treatment**

RNAi primers were designed using T7 promoter tags and used to PCR amplify ~300–500 bp of canoe or ran coding sequence. Transcription was performed using the Megascript T7 kit (Invitrogen) according to the manufacturer's protocol. Transfected S2 cells were seeded in 1 ml of serum-free Schneider's media in a 6-well dish at 10\(^4\) cells per well, and 120 \(\mu\)l RNA was incubated for 1 h, 2 ml of serum-containing growth media was added, and, 3 d later, expression was induced with CuSO\(_4\).

**Immunohistochemistry, imaging, and spindle angle measurements**

200 \(\mu\)l of clustered cells was seeded on 12-mm-diameter glass coverslips in a 24-well plate, allowed to adhere for 1 h, and encouraged to enter mitosis by addition of 300 \(\mu\)l of fresh growth media for 3–4 h. Adherent cells were fixed for 20 min with 4% formaldehyde in PBS followed by three rinses of wash buffer (0.1% saponin in PBS) and two rinses of block buffer (0.1% saponin and 1% BSA in PBS). The primary antibodies used were mouse anti-tubulin (1:2,000; Sigma-Addrich), rabbit anti–phosphohistone-3 (1:1,000; Millipore), mouse anti-Dlg (1:250; Developmental Studies Hybridoma Bank),
were taken with a confocal microscope (SP2; Leica) using an oil immersion 60× 1.4 NA objective. Spindle angles were defined as the angle between a line drawn perpendicular to the center of the Ed crescent and a line connecting the spindle poles.

Biochemistry

GST:Canoe and GST:Mud fusions were generated by cloning Canoe into the pGEX-4T1 vector using the 5′ EcoRI and 3′ NotI restriction sites and 5′ BamHI and 3′ SalI sites, respectively. Purified 6×-His–tagged Pins and Ran proteins were generated by cloning Pins and Ran into the pBH vector using the 5′ BglII and 3′ SalI restriction sites. Nucleotide exchange of

Figure 5. Canoe and RanGTP are required for Mud recruitment to Pins crescents. (A–C) Canoe and Ran are required for Mud recruitment to Pins. Ed:PinsTPR+LINKER fusions were expressed in S2 cells with no RNAi (A), canoe RNAi (B), or ran RNAi (C) and stained for endogenous Mud. (D and E) The Canoe RA domains are required for Mud recruitment to Pins. Ed:PinsTPR+LINKER fusions and GFP:Canoe proteins with or without RA domains were expressed in S2 cells and stained for Mud. (F–I) Canoe is not required for Mud recruitment to Pins (arrowhead). Ed:GFP or Ed:PinsTPR+LINKER fusions were expressed in S2 cells with no RNAi (F and G) or canoe RNAi (H) and stained for Mud. (I) MudTPR NLS localizes to Pins crescents independently of Canoe. Ed:PinsTPR+LINKER fusions and GFP:MudTIP-NLS were expressed in S2 cells in a wild-type (WT) and canoe RNAi background. Bars, 5 µm. 20 cell interfaces were analyzed for each experiment. (K, top) The importin-α/β (Impα/β) complex and Pins do not compete for Mud binding. A GST:MudTPR NLS fragment and purified His-tagged PinsTPR and importin-α/β proteins were incubated and probed for competition between PinsTPR and importin-α/β. Western blot using anti-His antibody shows presence of PinsTPR and importin-α/β. GST does not bind PinsTPR, importin-α, or importin-β (first through third lanes). GST:MudTPR NLS pulls down PinsTPR (2 µM) regardless of importin-α/β concentration. (bottom) Importin-β and Mud coimmunoprecipitate (IP). S2 cells were transfected with a GFP-tagged Mud containing the Pins-interacting domain and NLS (GST:MudTPR NLS) and the indicated FLAG or HA proteins (lanes 1–4). Only the positive control FLAG:PinsTPR and HA:importin-β can immuno precipitate GFP-MudTPR NLS (lanes 2 and 4).
purified Ran proteins was performed as previously described (Peterson et al., 2004). In brief, GMPNP or GDP was added to purified Ran protein at a threefold molar excess in 1 mM EDTA at room temperature for 30 min. Nucleotide exchange was quenched by addition of 10 nM MgCl₂. Purified 6×-His-tagged importin-α and β proteins were generated by cloning importin-α and β into the pET28b vector using the 5′ ‘Nhel and 3′ Nott restriction sites. All proteins were expressed in Escherichia coli BL21(DE3). For pull-down assays, GST fusions were added to glutathione agarose and rotated at 4°C for 30 min, washed three times in binding buffer (20 mM Heps, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 0.5% Tween 80), incubated with 50 µg of ligands in binding buffer, and rotated at 4°C for 1 h followed by washing, elution, and analysis by SDS-PAGE. For Western blots, His-tagged proteins were detected with a mouse penta-His antibody (1:1,000; Santa Cruz Biotechnology, Inc.).

For immunoprecipitations, S2 cells were lysed with NP-40 buffer (150 mM NaCl, 1% NP-40, 50 mM Triz, pH 8.0, and 1 mM PMSF) and passed through a 21-gauge needle. 5 µg of mouse anti-GFP antibody (Invitrogen), mouse anti-FLAG (Sigma-Aldrich), or mouse anti-HA (Roche) was added to lysates and gently mixed for 1 h at 4°C followed by washing, elution, and analysis by SDS-PAGE. For Western blots, the following antibodies were used: mouse anti-Flag (1:1,000; Sigma-Aldrich), mouse anti-Dlg (1:500; Developmental Studies Hybridoma Bank), rabbit anti-Canoe (1:200; Speicher et al., 2008), mouse anti-GFP (1:500; Invitrogen), and rabbit anti-Ran (1:1,000; Cell Signaling Technology).

Online supplemental material

Fig. S1 shows that RNAi depletion of Ran or Canoe does not significantly alter spindle morphology, centrosome number, or spindle length in S2 cells. Fig. S2 demonstrates the reduction in Dlg, Canoe, and Ran protein levels by Western blotting after RNAi. For pull-down assays, GST fusions were added to glutathione agarose and rotated at 4°C for 30 min, washed three times in binding buffer (20 mM Heps, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 0.5% Tween 80), incubated with 50 µg of ligands in binding buffer, and rotated at 4°C for 1 h followed by washing, elution, and analysis by SDS-PAGE. For Western blots, the following antibodies were used: mouse anti-Flag (1:1,000; Sigma-Aldrich), mouse anti-Dlg (1:500; Developmental Studies Hybridoma Bank), rabbit anti-Canoe (1:200; Speicher et al., 2008), mouse anti-GFP (1:500; Invitrogen), and rabbit anti-Ran (1:1,000; Cell Signaling Technology).

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Figure S1. **Ran depletion does not alter spindle morphology, centrosome number, or spindle length in S2 cells.** S2 cells treated with ran RNAi, cno RNAi, or untreated (wild type). (A) Centrosome number determined by counting γ-tubulin–positive foci [SEM measured for five repeated experiments]. WT, wild type. (B) Mitotic spindle length determined by measuring the centrosome-to-centrosome distance after γ-tubulin staining. (C) Spindle morphology determined by α-tubulin staining (n = 30). Bar, 5 µm.

Figure S2. **Reduction in Dlg, Canoe, and Ran protein levels after dlg, cno, or ran RNAi in S2 cells.** S2 cells treated with the indicated RNAi (+) or untreated (−) and Western blotted for the respective protein. Note the strong reduction in Ran protein and the intermediate reduction in Canoe and Dlg proteins. α-Tubulin protein is used for a loading control.