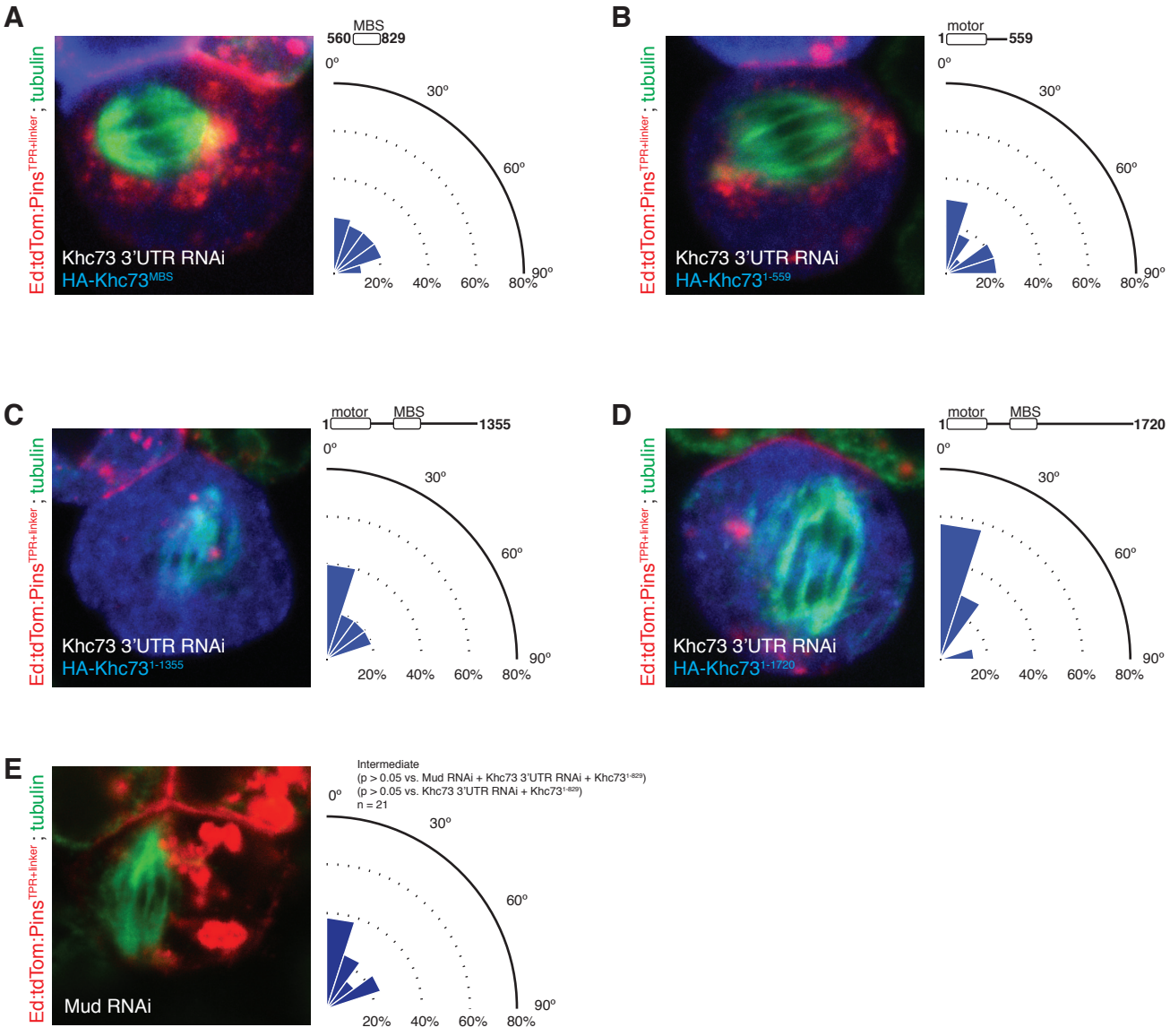
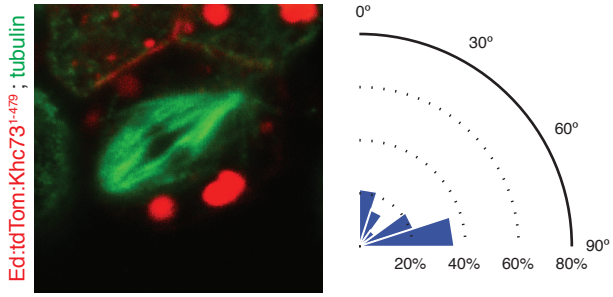
SUPPLEMENTAL FIGURES

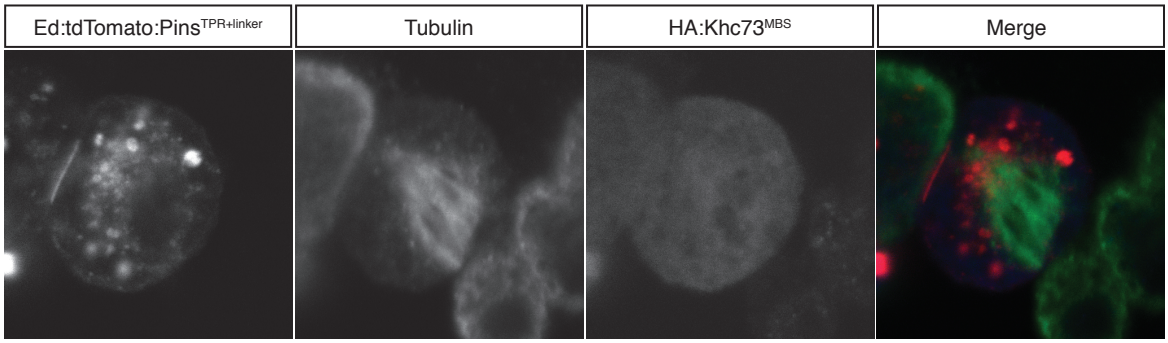
Lu and Prehoda 2013 **Figure S1**



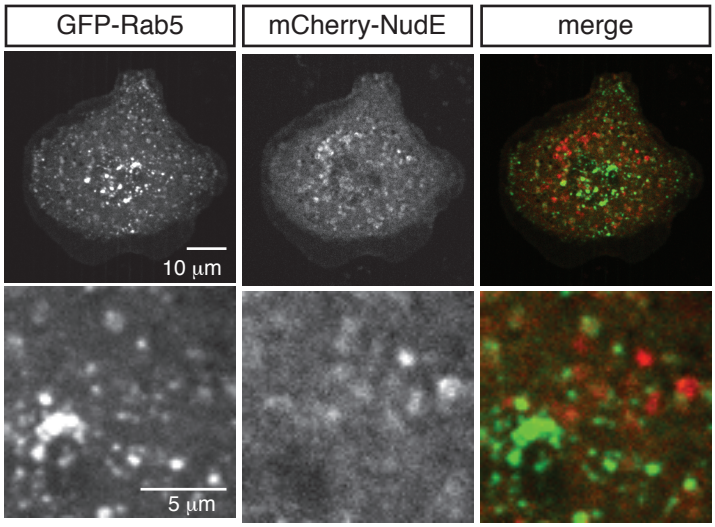
A


Ed:tdTom:Khc73$^{1-479}$; tubulin
0°
30°
60°
90°
20%
40%
60%
80%

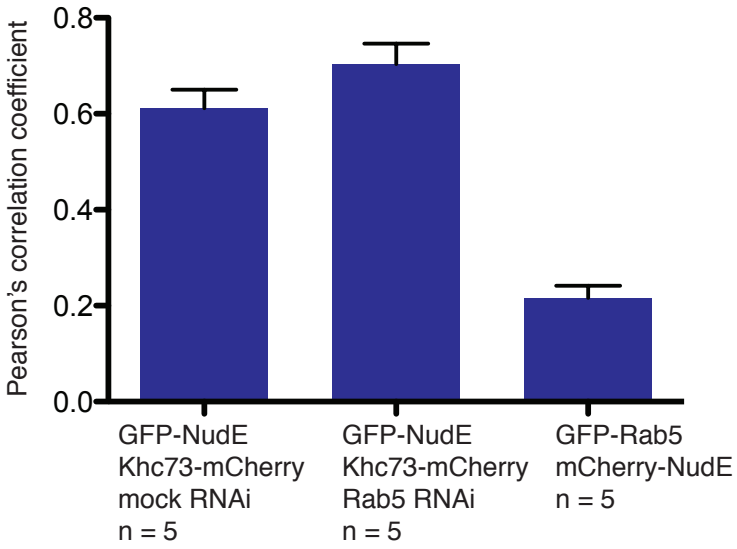
B


Ed:tdTomato:Pins$^{TPR+linker}$
Tubulin
HA:Khc73^{MBS}
Merge

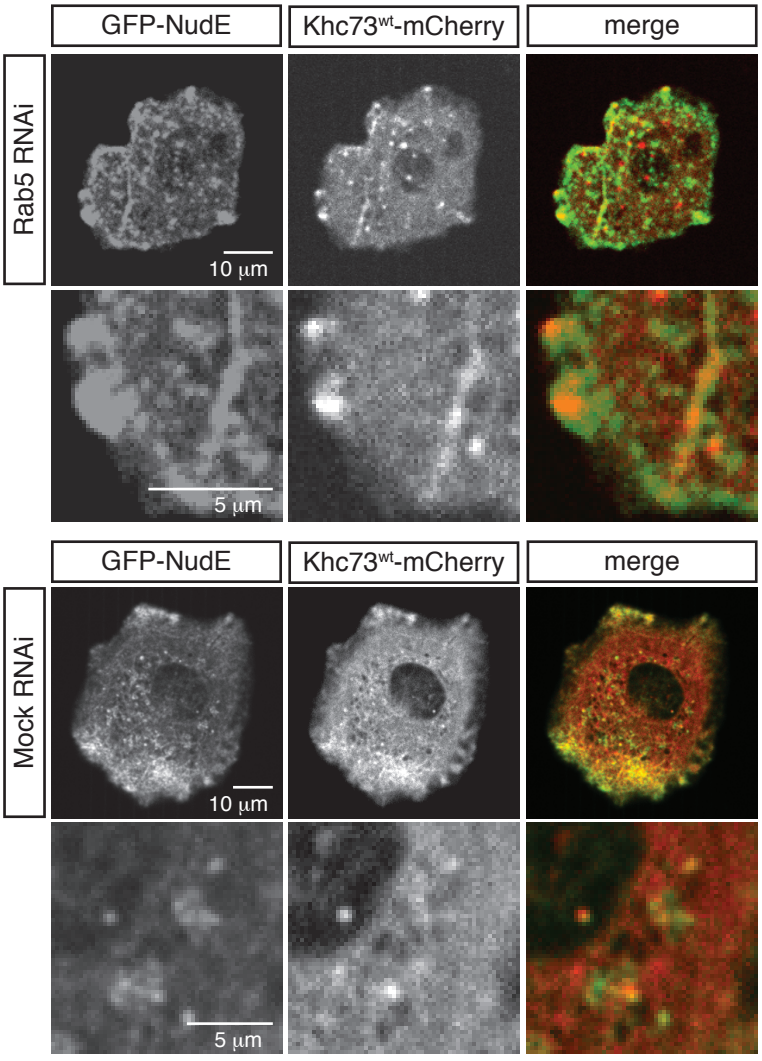
A


GFP-Rab5
mCherry-NudE
merge
10 μm
5 μm

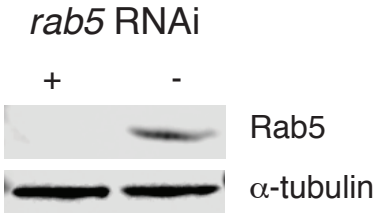
C


Pearson's correlation coefficient
0.8
0.6
0.4
0.2
0.0
GFP-NudE
Khc73-mCherry
mock RNAi
n = 5
GFP-NudE
Khc73-mCherry
Rab5 RNAi
n = 5
GFP-Rab5
mCherry-NudE
n = 5

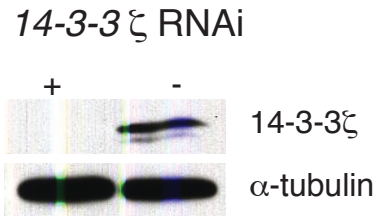
B


Rab5 RNAi
GFP-NudE
Khc73wt-mCherry
merge
10 μm
5 μm
Mock RNAi
GFP-NudE
Khc73wt-mCherry
merge
10 μm
5 μm

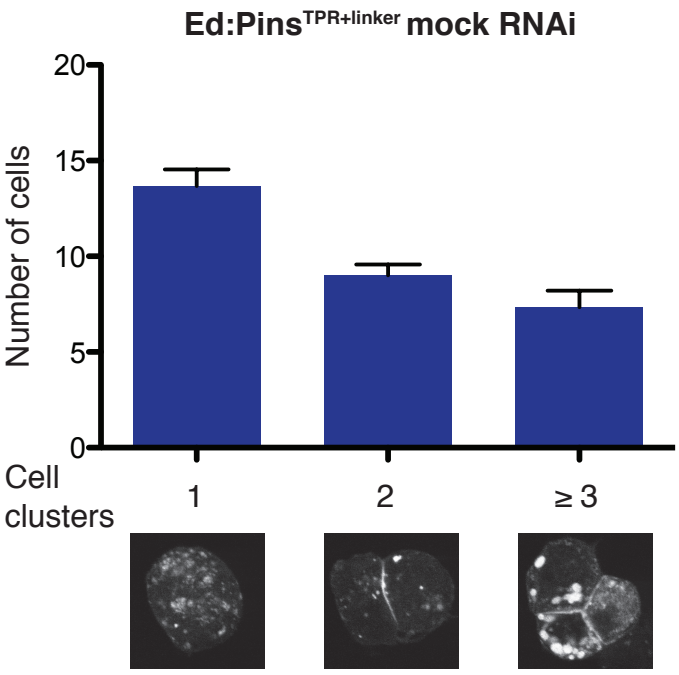
D


rab5 RNAi
+
-
Rab5
α-tubulin

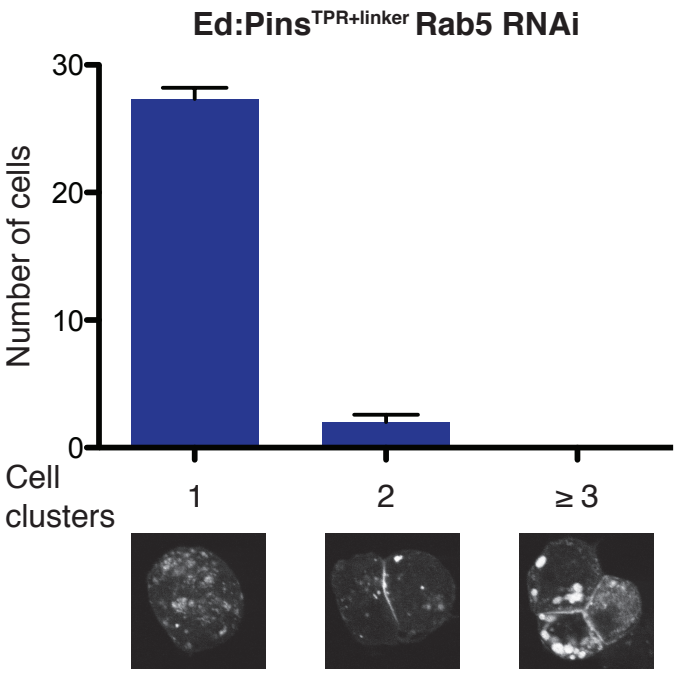
E


14-3-3 ζ RNAi
+
-
14-3-3ζ
α-tubulin

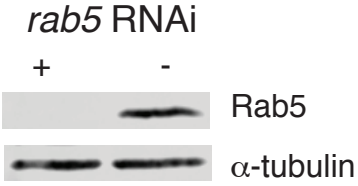
A


Ed:Pins^TPR+linker mock RNAi
Number of cells
20
15
10
5
0
Cell clusters
1
2
≥ 3

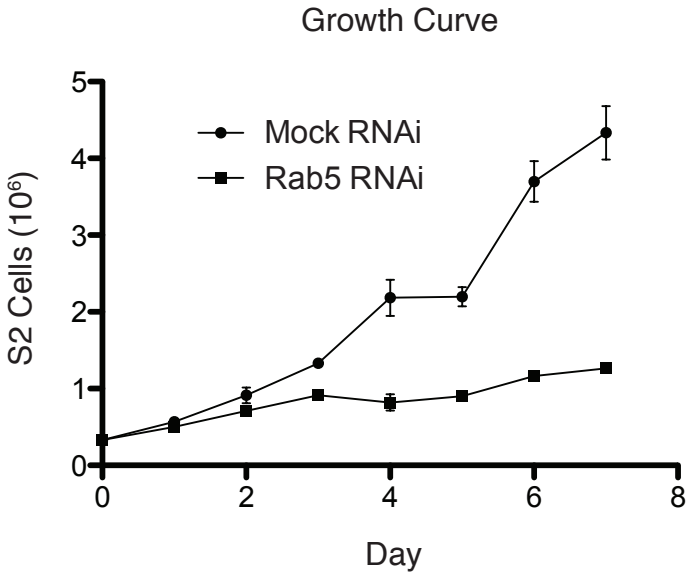
B


Ed:Pins^TPR+linker Rab5 RNAi
Number of cells
30
20
10
0
Cell clusters
1
2
≥ 3

C


rab5 RNAi
+
-
Rab5
α-tubulin

D


Growth Curve
Mock RNAi
Rab5 RNAi
S2 Cells (10^6)
5
4
3
2
1
0
0
2
4
6
8
Day

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. The minimal domains required for spindle orientation rescue include the Motor and MBS domains togther.

(A) The Motor and (B) MBS domains alone are not sufficient for restoring any part of the Pins$^{TPR+linker}$ spindle orientation activity, however, rescue constructs containing both domains (C, D) restore activity in part or in full.

(E) Ed:Pins$^{TPR+linker}$ with Mud RNAi control for conditions of figures 1F and 1G. $P > 0.5$ vs. Ed:Pins$^{TPR+linker}$ + Khc73 RNAi + Khc73$^{1-829}$ (figure 1F) and $P > 0.5$ vs. Ed:Pins$^{TPR+linker}$ + Khc73 RNAi + Mud RNAi + Khc73$^{1-829}$ (figure 1G).

Figure S2, related to Figure 2. Ed-motor has no activity, MBS does not colocalize with Ed-Pins.

(A) The motor domain of Khc73 is not sufficient for spindle orientation in the Ed-induced spindle orientation assay. S2 cells expressing Ed:tdTomato:Khc73^{motor} do not bias spindle orientation.

(B) Khc73's Maguk Binding Stalk (MBS) does not localize to Ed-Pins$^{TPR+linker}$ crescents.

Figure S3, related to Figure 4. Rab5 does not play a role in NudE/Khc73 co-localization.

(A) Still frame from live-imaging of S2 cells expressing GFP-Rab5 and mCherry-NudE reveals that NudE does not localize to Rab5-containing vesicles. S2 cells were plated on ConA-coated chambers.

(B) Still frame from live-imaging of S2 cells expressing GFP-NudE and Khc73-mCherry in a mock RNAi and Rab5 RNAi background. It appears that Rab5 RNAi slightly improves NudE/Khc73 overlap. S2 cells were plated on ConA-coated chambers.

(C) Quantification of GFP-Rab5 and mCherry-NudE co-localization demonstrates that these two components do not significantly overlap. Quantification of GFP-NudE and Khc73-mCherry in mock RNAi or Rab5 RNAi conditions reveals that Rab5 knockdown does not significantly affect the co-localization NudE and Khc73. n=5, mean ± S.D. shown.

(D) Western blot shows knockdown of endogenous Rab5 from S2 cells expressing GFP-NudE and Khc73-mCherry treated with Rab5 RNAi (left lane) compared to cells treated with mock RNAi (right lane). 20 μg total lysate was loaded, α-tubulin shown to demonstrate equal loading.

(E) Western blot shows knockdown of endogenous 14-3-3ζ. 30μg lysate loaded, α-tubulin used to demonstrate equal loading.

Figure S4, related to Figure 5. Rab5 RNAi interferes with the induced polarity spindle orientation assay.

(A) Mock RNAi treatment to S2 cells does not interfere with induction of Ed:Pins$^{TPR+linker}$ crescents. Over half of all cells expressing Ed:Pins$^{TPR+linker}$ contain one or more Ed:Pins$^{TPR+linker}$ score-able crescents. An n=30 was collected for three independent trials. Error bars represent S.E.M.

(B) Rab5 RNAi treatment to S2 cells expressing Ed:Pins$^{TPR+linker}$ inhibits Ed:Pins$^{TPR+linker}$ crescent formation. Less than 10% of cells expressing Ed:Pins$^{TPR+linker}$ in Rab5 RNAi conditions display Ed crescents, presumably because endocytic trafficking is essential for cortical expression of Ed. An n=30 was collected for three independent trials. Error bars represent S.E.M.

(C) Western blot shows knockdown of endogenous Rab5 from S2 cells expressing Ed:Pins$^{TPR+linker}$ treated with Rab5 RNAi (left lane) compared to cells treated with mock RNAi (right lane). 20 μg total lysate was loaded, α-tubulin shown to demonstrate equal loading.
(D) Rab5 RNAi treatment (closed squares) severely inhibits S2 cell growth compared to mock RNAi treatment (closed circles) over the course of seven days. Mean and standard deviation from three independent trials of each condition are reported.

Movie S1, related to figure 2. Khc73 motility on microtubules.
Khc73-mCherry (red) rapidly moves on microtubules (GFP-αtubulin; green) to the periphery of the cell supporting the idea that it directly interacts with tubulin through its motor domain.

Movie S2, related to Figure 2. Khc73 moves towards plus ends of MTs and stops at ends, marked by EB1.
Khc73-mCherry (red) moves along microtubules to the periphery of the cell and stops at microtubule plus ends (marked by GFP-EB1), where it remains in proximity to the cell cortex.

Movie S3, related to Figure 4. Khc73 and NudE comigrate in S2 cells.
Khc73-mCherry (red) and GFP-NudE (green) move together toward the periphery of the cell. Interestingly, Khc73 sometimes appears as a comet tail.

Movie S4, related to Figure 4. Khc73 and NudE travel in opposite directions along a linear path.
Khc73-mCherry (red) and GFP-NudE (green) can be observed travelling in opposite directions along a linear path, presumably along microtubules, suggesting that NudE is not solely trafficked by the plus-end directed Khc73, but also by minus-end directed Dynein.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Khc73 sequence

For this study we cloned, in its entirety, Khc73 from a cDNA library generated from *Drosophila* S2 cells. This sequence differs slightly from the sequence deposited in Genbank (Accession: NP_609201.3). The Khc73 sequence used in this study notably lacks an unconserved region in the C-terminal stalk, near the 14-3-3 binding site. The following is the DNA sequence for this clone of Khc73:

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GCCACTGGAAAAAATAGAGAGAAAACAACCAAAGACATTCGCATTCGATCACTGCTTTTACT
CATTGAACCCCGAGGACGAGAACTTTGCGTCCCAGGAGACAGTGTTCGATTGCGTGGGAC
GTGGAATTCTGGATAATGCATTCCAGGGCTATAATGCGTGCATATTCGCTTACGGCCAGAC
AGGTTCTGGCAAGTCCTACACGATGATGGGCACCCAGGAGAGCAAGGGCATCATTCCACG
TCTGTGTGACCAACTCTTCTCGGCCATAGCCAACAAATCCACACCCGAGCTTATGTACAAG
GTGGAGGTGTCCTACATGGAGATCTATAACGAGAAGGTCCACGATCTGCTCGATCCCAAGC
CGAACAAACAGTCGCTTAAGGTGCGCGAGCACAATGTTATGGGCCCATATGTGGACGGACT
GTCGCAGCTGGCTGTGACATCCTATCAGGACATCGATAACCTCATGACCGAAGGCAACAAG
TCACGAACGGTGGCCGCCACCAATATGAATGCCGAGTCGTCGCGCTCCCACGCCGTCTTC
TCAGTGGTCCTCACTCAGATACTCACGGATCAGGCGACGGGAGTGAGCGGCGAGAAGGTG

TCCCGCATGTCCCTGGTGGATTTGGCTGGCTCCGAGCGGGCTGTGAAAACGGGAGCTGTT
GGCGATCGTCTCAAGGAAGGCTCTAACATCAACAAATCTCTGACCACACTTGGCCTGGTCA
TCTCCAAGTTGGCCGATCAATCCAATGGCAAGAAGAGCGGTAACGACAAATTCGTTCCCTA
CCGCGATTCCGTGCTCACCTGGCTGCTGAAAGACAATTTGGGTGGCAATTCCAGAACTGTT
ATGGTAGCGACAATCTCGCCATCGGCAGATAACTACGAGGAGACGCTTTCCACGCTGCGTT
ATGCAGATCGAGCCAAGCGCATTGTTAACCACGCTGTTGTCAACGAAGATCCCAATGCCCG
CATCATTCGTGAGCTGCGACACGAGGTGGAGACCCTTAGAAGTATGCTGAAACACGCCACT
GGTTCACCAGTGGGCGATGTCCAGGATAAGCTGGCTGAGAGCGAGAACCTGATGAAACAG
ATTTCGCAGACTTGGGAGGAGAAGCTGGTTAAGACAGAACGCATTCAAAACGAACGGCAGC
AGGCTCTAGAGAAAATGGGCATTAGTGTACAGGCCAGTGGCATCAAGGTAGAGAAGAACAA
GTACTACTTGGTCAATTTAAATGCTGATCCGTCCCTTAACGAGCTGCTGGTCTATTATCTGA
AGGATCGCACACTGATTGGCGGACGCACTATCAGTGGCCAGCAGCCGGATATACAACTTTC
CGGTCTCGGAATCCAGCCCGAGCACTGTGTGATCACTATCGAGGATAGTGGACTGTACATG
GAGCCAGTGCAGGGAGCGCGTTGCTTTGTGAACGGATCTGCTGCTGTGGAAAAGACACCA
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CAGGAGTACGAGCGTCAGTTCCAGCAGCTACGCAATATTCTGTCGCCTAGTACACCATATG
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CAAATGCGAGTGGAAAAGTGGGCACAGGAACGAGATGAGATGTTTCGTCGCAGTTTGGGT
CAGCTGAAAACGGATATTATGCGTGCGAATTCCCTGGTCCAGGAGGCCAACTTTCTGGCTG
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GAAGACGAGTTCGATGACAAGGACGAGGATAATGCCAAGCCACAGGATCCGTTTTACGAGT
CGCAAGAGAACCACAATCTTATTGGCGTGGCCAATATATTCTTGGAGGTTCTGTTTCATGAC
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TGTCTTTTGCCAGTACACTTTTTGGGGTCACCAAGAGATGGTTGTTCCGGTTATCAATGCTG
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AATTCCGTCTTGTGCGGGGAGAGAACGCCATCTGGCAGAGCGGTGTCACCTATGAGGTGG
TCTCTAACATTCCAAAAGCCTCCGAGGAGTTGGAGGACCGCGAGTCTCTTGCACAGTTGGC
TGCCAGCGGAGATGATTGCTCCGCAAGTGACGGCGAAACCTACATAGAGAAATACACGCG
CGGTGTTTCGGCGGTGGAGAGCATACTGACTCTGGATAGACTGCGGCAGAATGTGGCGGT
CAAGGAGCTGGAAACGGCACATGGACAGCCGCTCAGCATGCGCAAGACCGTCAGCGTGCC
GAACTTCTCACAGATTATGCGCTTCGATGCATCGATGGAGTCGCTGCTGAATGTGGGACGA
TCCGAGTCCTTTGCCGATCTCAATAACAGCGCTTTGGGCAACAAGTTTACTCCAGCAGGTC
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GGGCATGCGCATGACTACGTTGCACGAGGAGCCACTGGGTGGACACAGATCTCTCGACGA
AGAGCCTGAGGACAGCTACAGCGACTCGGAATATGCTGCCGAGTACGAACAGGAGCGGCA
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GAGTCAGTGTTAATCCGGCCGTATAACACCAGCGGCGTCATTCGCTTTGTGGGAACCACGG
AATTCCAACCCGGTGCCTGGATTGGCGTTGAATTGGACACACCAACGGGCAAAAACGACG
GCAGCGTGAAGGGCGTTCAGTATTTCCAGTGCAAGCCCAAGCACGGCATGTTTGTGCGCT
CCGACAAGCTGATGCTGGACAAGCGTGGCAAGGCGATGCGAGCCTACAAGGCCGCCGAG
AAGAGCAACAGCATCAGCAAAGAGATGAGTACCTCGATGACTGGCTCGATGACACGCTCCA
AGAGCCGCGGCGATTCGCTAAACCTTTCGGCGCGTAAATGA

RNAi in S2 cells

RNA interference was performed as follows. Primers for dsRNAs of ~150-400bp with T7 promoter sites targeted to Khc73's 3'UTR, Par-1, NudE, Mud, 14-3-3ζ, and 14-3-3ε were designed using SnapDragon ds-RNA Design service at http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl. PCR-amplified templates were transcribed and purified using the Megascript T7 kit (Ambion, Austin, TX). S2 cells were transfected as described above and incubated for 24 hours before being seeded at a density of 1 x 10^6 cells per well in 1 mL of serum-free media. 10-100 μg dsRNA was added to the cells and allowed to incubate at room temperature for 1 hr before being supplemented with 2mL serum-containing media. RNAi was induced for 72-96 hrs before fixing.

Determining RNAi efficiency in S2 cells

RNAi efficiency was determined by Western blotting for 14-3-3ζ, RNAi and Rab5 RNAi. For these two conditions, transfected/RNAi-ed S2 cells were lysed in 20mM Tris pH 8.0, 150mM NaCl, 1% NP-40, containing Complete protease inhibitor cocktail (Roche). 20-30μg of cell lysate were probed for 14-3-3ζ and Rab5, using α-tubulin as a loading control. Antibodies were used as follows: 1:1000 Rabbit anti-14-3-3ζ (Millipore), 1:1000 Rabbit anti-Rab5 (AbCam), 1:1000 Mouse-anti-DM1A (Santa Cruz).

To determine Khc73 RNAi efficiency for the induced polarity spindle orientation assays, because we could not procure an antibody to Khc73, we verified knockdown of Khc73 by including a Khc73 3'UTR RNAi-only control with every rescue experiment conducted. Khc73 RNAi leads to complete loss of spindle orientation, and only rescue experiments in which the Khc73 RNAi control exhibited random spindle orientation would be analyzed for rescue. Because Khc73 RNAi leads to complete loss of spindle orientation, we conclude that th

Primers used to generate dsRNA for RNAi experiments in S2 cells
Khc73 3'UTR Forward: 5' taatacgactcactatagggGACTCGACAAGCAACGAACA 3'
Khc73 3'UTR Reverse: 5' taatacgactcactatagggAACGCTTCATTCCAAAATCG 3'
14-3-3 ζ Forward: 5' taatacgactcactataggGGGACAACCTGACTCTCTGG 3'
14-3-3 ζ Reverse: 5' taatacgactcactataggGCTTTGCTGGTTGCGTTTTA 3'
14-3-3 ε Forward: 5' taatacgactcactataggCGATCGAACAGAAGGAGGAG 3'
14-3-3 ε Reverse: 5' taatacgactcactataggg ACACCGAGAAGTTCAATGCC 3'
NudE Forward: 5' taatacgactcactataggg CCGCGAAATGGAAATAGAAA 3'
NudE Reverse: 5' taatacgactcactataggg CACTGGGGTAAAGCGTGACT 3'
Par-1 Forward: 5' taatacgactcactataggg TCAAAGCTGGACACGTTCTG 3'
Par-1 Reverse: 5' taatacgactcactataggg GTAGCCCATCGCGACTAGAG 3'
Rab5 Forward: 5' taatacgactcactataggTCAGGACAGTTTTCAGCGTG 3'
Rab5 Reverse: 5' taatacgactcactataggGTTCGTCGGTCGATTTGTTT 3'
Mud primer sequences published in Johnston et. al. 2009

Colocalization quantification

S2 cells expressing GFP-NudE and Khc73^{FL}-mCherry or Khc73^{S1374A}-mCherry were plated on ConA-coated chambers for 1-2 hours and live-imaged by confocal microscopy using a 100X objective, as described in the main Experimental Procedures section. Still frames from 5 individual cells were analyzed using ImageJ with the LoG 3D plugin, using sigma value 2 or 3 for both GFP-NudE and Khc73^{FL}-mCherry or Khc73^{S1374A}-mCherry channels to enhance punctae. The enhanced images were then analyzed for colocalization (Pearson's correlation coefficient) using the JACoP colocalization plugin for ImageJ.

Bacterial Protein Purification

All bacterially-purifiable proteins were expressed in the *E. coli* BL21(DE3) strain induced with 250 μM IPTG. For His-tag protein purification, 14-3-3ζ and Khc73 (amino acids 580-829) were cloned into a pBH4-based vector containing a cleavable N-terminal 6x Histidine epitope. His-tagged proteins were affinity purified using Ni-NTA resin (QIAGEN, Germantown, MD). For GST-fusion proteins, Dlg isoform G (amino acids 771-975), Khc73 (amino acids 1336-1447), Khc73 (amino acids 1336-1447 S1374D), and 14-3-3ε, were cloned into the pGEX-4T vector

(GE Lifesciences). GST-tagged proteins were affinity purified on glutathione-agaroes (Invitrogen) and eluted with 10mM reduced glutathione.

Cell clustering assay

To determine whether Rab5 RNAi interfered with cortical Ed:Pins$^{TPR+linker}$ expression, thereby impeding its function to polarize Pins$^{TPR+linker}$, we quantified the number of intercellular adhesion events observed for S2 cells expressing Ed:Pins$^{TPR+linker}$ in mock RNAi and Rab5 RNAi conditions. S2 cells were transfected with Ed:Pins$^{TPR+linker}$, treated with Rab5 RNAi, and shaken at 175rpm to induce polarization. Cells were fixed, stained, and imaged by confocal microscopy (see materials and methods). Antibody used: 1:1000 mouse anti-DM1A (Santa Cruz). n=30 were collected from S2 cells expressing Ed:Pins$^{TPR+linker}$ from three independent trials and scored for non-clustered (i.e. individual cells), cell clusters of 2, or cell clusters of 3 or more.

Growth assay

To determine the effects of Rab5 RNAi on S2 cell growth, the cell densities of mock RNAi- or Rab5-RNAi treated S2 cell cultures were monitored for seven days. 1mL S2 cells (at a density of 10^6 cells/mL) were seeded in serum free Schneider's S2 media in a well of a 6-well plate, and either water (mock RNAi) or Rab5 dsRNA were added to the well, and allowed to incubate at room temperature for 1 hour. 2mL Schneider's S2 media containing 10% FBS were added to each well, making the final S2 cell density 0.33 x 10^6 cells/mL. Cell density was measured every 24 hours. Three independent trials were conducted for each condition.