Supporting information

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SI Materials and Methods

Immunocytochemistry. All cells used in immunocytochemistry experiments were harvested 72 h after transfection for RNAi and plated on glass coverslips for 1 h. Cells were then fixed with 4% paraformaldehyde in buffer (60 mM PIPES, 25 mM Hepes, 10 mM EGTA, 4 mM MgCl2). Cells were permeabilized in PBS with 1% Triton X-100 and 3% BSA and washed three times with PBS 1× with 0.1% Triton X-100 and 1% BSA (PBSTB). Antibody incubations were carried out overnight at 4 °C. Secondary antibody incubations were for 2 h in PBSTB. DNA was stained using DAPI in Vectashield mounting medium H-1200 (Vector Laboratories). To stain cells with the lipophilic dye FM4-64 red (Molecular Probes), cells were cultured in medium containing the dye for 24 h and then fixed and stained. The water-soluble FM dye (Invitrogen), which is nontoxic to cells and virtually nonfluorescent in aqueous media, is inserted into the outer leaflet of the membrane, where it becomes intensely fluorescent. Primary antibodies used were rat antitubulin antibody (clone YL1/2, dilution 1:50; Sigma-Aldrich), mouse antitubulin (clone DM1A, dilution 1:500; Sigma-Aldrich), mouse anti–γ-tubulin clone (GTU88, dilution 1:100; Sigma-Aldrich), rabbit anti-BubR1 (dilution 1:100), chicken anti-CID (dilution 1:5,000) (1), T47 mouse anti-Lamin (dilution 1:50) (2), rabbit anti-mushroom body defect (Mud; dilution 1:500; a gift from H. A. Nash, National Institutes of Health, Bethesda, MD), and rabbit anti-Rab5 (dilution 1:500; Abcam). Immunostaining with the mouse 3F3/2 antibody (dilution 1:500; Boston Biologicals) was performed according the work by Maresca and Salmon (3). Alexa-conjugated secondary antibodies green- or red-conjugated anti-rat, anti-mouse, anti-rabbit, and anti-chicken were from Molecular Probes (Invitrogen).

Microscopy and Live Imaging. Mitotic analysis of cellular structures was performed using an inverted Zeiss Axiovert 200 microscope with a 100× (NA 1.4) lens and a Cool Snap HQ camera (Photometrics) running Metamorph software (Media Cybernetics). For time-lapse experiments, DMel cells were plated on No. 1 0.5-thickness coverslips 72 h after RNAi treatment. Imaging was performed on a Zeiss Axiovert 200 microscope with a 100× (NA 1.4) lens. Specimens were maintained at 25 °C using combined stage and objective heating units (Tempcontrol 37–2 digital; Zeiss). At 30-s or 1-min intervals, a *z* series of 10 1-mm sections was captured using a Perkin-Elmer Ultraview RS spinning disk confocal head. All images shown are maximum intensity projections. Data stacks were deconvolved using the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V.). Images were processed, and figures were generated in Adobe Photoshop software.

ImageJ software version 1.42q (National Institutes of Health) was used to calculate the length of interkinetochore distance after RNAi treatments. For each of centromere/kinetochore pair, a straight line was drawn and measured using the line tool.

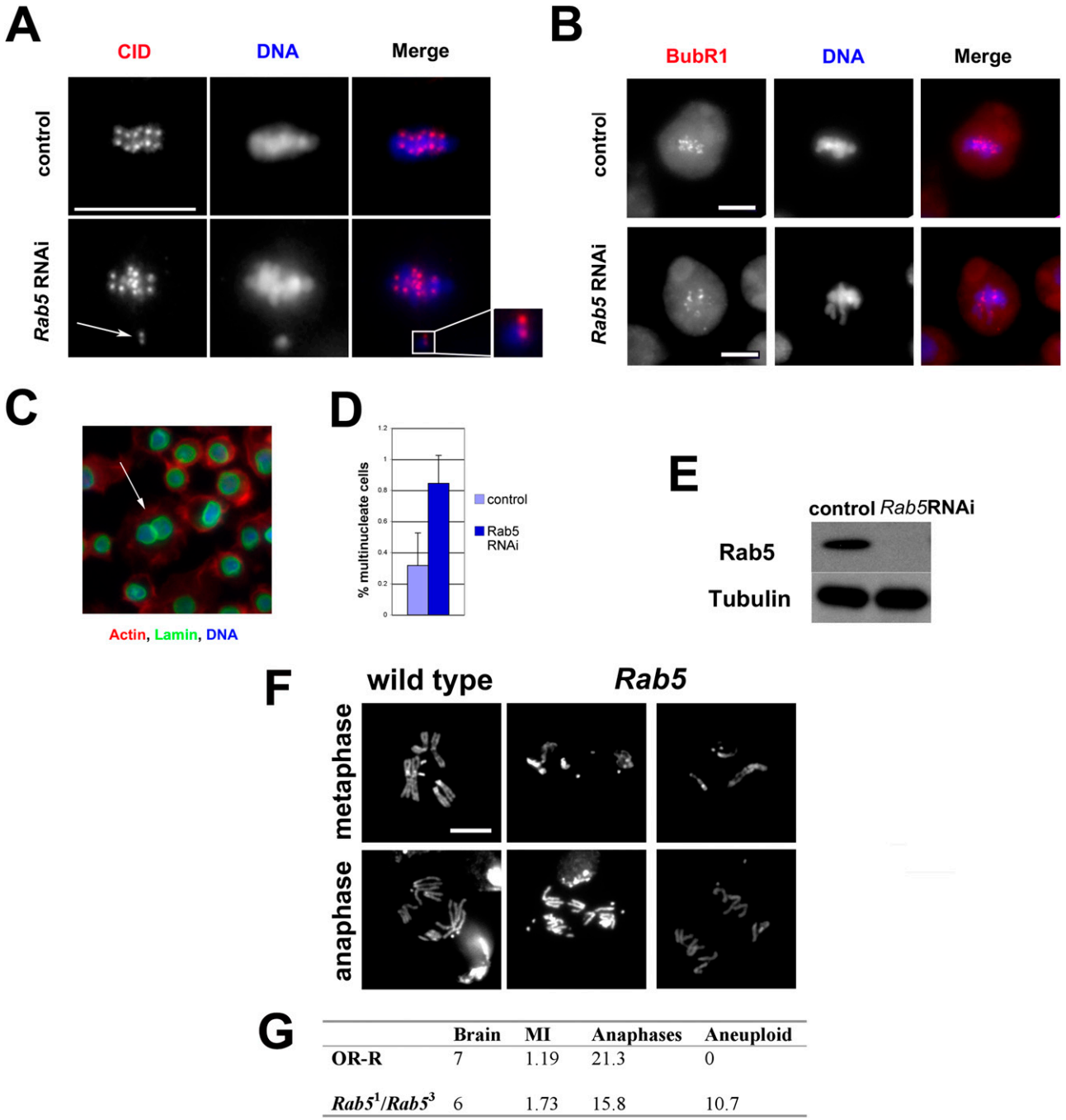
Protein Affinity Purifications, MS, and Western Blot Analysis. Protein-A (PtA) affinity purification was performed as previously described (4, 5) with the exception of the addition of Guanosine 5′-O-(γ-thio) triphosphate (GTPγS) to the cell extract at a concentration of 0.5 mM. Proteins were separated on either 4% to 20% or 8% to 16% acrylamide gels for quality control, but then, gel-free samples were methanol/chloroform-precipitated and analyzed by liquid chromatography MS as described previously (4, 5). The MS/MS fragmentation data achieved were used to search the National Center for Biotechnology Information and FlyBase databases using the MASCOT search engine (http://www.matrixscience.com). Probability-based MASCOT scores were used to evaluate identifications. Only matches with $P < 0.05$ for random occurrence were considered significant.

For Western blots, total cellular extracts were resolved on 8% to 16% gradient SDS/PAGE gels, transferred onto PVDF membranes, and probed with mouse anti-Polo (dilution 1:200; M294), mouse antidynein heavy chain (Dhc; dilution 1:2,000; a gift of T. S. Hays, Vanderbilt University Medical Center, Nashville, TN), rabbit anti-Rab5 (dilution 1:2,000; Abcam), rabbit anti-Mud (dilution 1:2,000; a gift from H. A. Nash, National Institutes of Health, Bethesda, MD), mouse monoclonal anti-Myc (clone 9E10; Santa Cruz), and rabbit anti-nonclaret disjunctional (Ncd) (dilution 1:2,000; a gift from P. G. Wilson, Georgia University, Atlanta, GA). Immunoreactive protein bands were detected using ECL Western Blotting Detection System (Amersham).

In Vivo Pull-Down Assay and Immunoprecipitation from DMel Cells. Cells stably expressing either PtA::Lamin or PtA alone were transfected with a plasmid encoding a Myc-tagged Rab5 or Lamin transgene and incubated at 25 °C for 42 h. The Gateway vector pAMW contains 6×Myc epitopes (Drosophila Genomics Resource Center). The proteasome inhibitor MG132 (Sigma) at a concentration of 25 μM and GTPγS (BIOMOL) at a concentration of 0.5 mM were added to the medium 6 h before harvesting the cells. Cells were then collected and stored at −80 °C. The cell pellet was resuspended in 0.5 mL extraction buffer (50 mM Hepes, pH 7.5, 100 mM KAc, 50 mM KCl, 2 mM MgCl2, 2 mM EGTA, 0.1% Nonidet P-40, 5 mM DTT, 5% glycerol, Roche Complete Protease Inhibitors) and homogenized using a high-performance disperser (Fisher). Homogenates were centrifuged at 6,797 × *g* at 4 °C in an Eppendorf 5417R centrifuge for 20 min, and supernatants were transferred into new tubes; 50 μL Dynabeads (Invitrogen) conjugated to rabbit IgG (MP Biochemicals) were added to the supernatants and incubated for 2 h on a rotating wheel at 4 °C. Beads were then washed five times for 5 min in 1 mL extraction buffer. Proteins were eluted from beads with 0.5 mL elution buffer (0.5 M NH_4OH, 0.5 mM EDTA, pH 8.0), lyophilized, and resuspended in Laemmli SDS/PAGE sample buffer (Sigma). For immunoprecipitation, we used DMel cells transfected with Myc::Rab5 and Myc::Lamin as described above. Cell pellets were resuspended in extraction buffer and lysed as described above. To the clarified supernatants, we added 15 μL anti-Mud antibody or 100 ngr rabbit IgG and incubated for 1 h at 4 °C on a rotating wheel. Then, we added 50 μL prewashed Protein A-DynaBeads (Invitrogen) and incubated for 2 h at 4 °C on a rotating wheel. Subsequent steps were similar to the pull-down experiments, except that, at the end, the beads were resuspended in 30 μL 1× Laemmli sample buffer (Sigma) and boiled for 10 min. Proteins were separated on an SDS/PAGE gel, transferred onto a PVDF membrane, and probed to detect the antigens shown in Fig. 3 *A* and *B*.

Statistical Analysis. Statistical analysis was performed using Excel software. Values are given as SD in all figures. Quantification of the binucleate phenotype in *Rab5* RNAi and control cells was carried out manually on images collected using Metamorph software (Media Cybernetics). Interkinetochore distances in micrometers were grouped and displayed as distribution plots using Excel software. For 3F3/2 quantification, we used ImageJ and Excel software, and it is described in Fig. 6.

1. Przewloka MR, et al. (2007) Molecular analysis of core kinetochore composition and assembly in *Drosophila melanogaster*. *PLoS One* 2:e478.
2. Paddy MR, Belmont AS, Saumweber H, Agard DA, Sedat JW (1990) Interphase nuclear envelope lamins form a discontinuous network that interacts with only a fraction of the chromatin in the nuclear periphery. *Cell* 62(1):89–106.
3. Maresca TJ, Salmon ED (2009) Intrakinetochore stretch is associated with changes in kinetochore phosphorylation and spindle assembly checkpoint activity. *J Cell Biol* 184: 373–381.
4. Chen F, et al. (2007) Multiple protein phosphatases are required for mitosis in *Drosophila*. *Curr Biol* 17:293–303.
5. D'Avino PP, et al. (2009) *Isolation of Protein Complexes Involved in Mitosis and Cytokinesis from Drosophila Cultured Cells. Methods in Molecular Biology: Mitosis* (Humana Press, Totowa, NJ).



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	Brain	MI	Anaphases	Aneuploid
OR-R	7	1.19	21.3	0
$Rab5^{1}/Rab5^{3}$	6	1.73	15.8	10.7

Fig. S1. (*A*) Depletion of Rab5 leads to chromosome alignment and cytokinesis defects. Cells were treated with *Rab5* dsRNA (*Rab5* RNAi) or *GFP* dsRNA (control) for 3 d and fixed and stained to detect CID (red) and DNA (blue). *Inset* shows a 2× magnification of CID-positive dots on the misaligned chromosome indicated by the arrow. (Scale bar: 10 μm.) (*B*) Cells were treated as in *A* and stained to reveal BubR1 (red) and DNA (blue). (Scale bars: 10 μm.) (*C*) *Drosophila* S2 cells were treated with dsRNA against *Rab5* for 72 h and then fixed and stained to reveal actin (red), lamin (green), and DNA (blue). The arrow indicates a binucleate cell. (*D*) Proportion of multinucleate cells in control and *Rab5* RNAi cells in three independent experiments. More than 2,000 cells were counted for each experiment. Bars indicate SDs. (*E*) Western blot analysis of Rab5 and tubulin (loading control) levels in extracts from cells treated with dsRNAs against *Rab5* (*Rab5* RNAi) or *GFP* (control). (*F*) *Rab5* mutant neuroblasts display abnormal metaphases and anaphases. Third instar larval brains from wild type (Oregon-R) and $Rab5^{1}/Rab5^{3}$ mutants were squashed, fixed, and stained with DAPI to visualize DNA. (*G*) A table showing a quantification of the mitotic index (MI), anaphases, and aneuploid cells is shown. (Scale bar: 5 μm.)

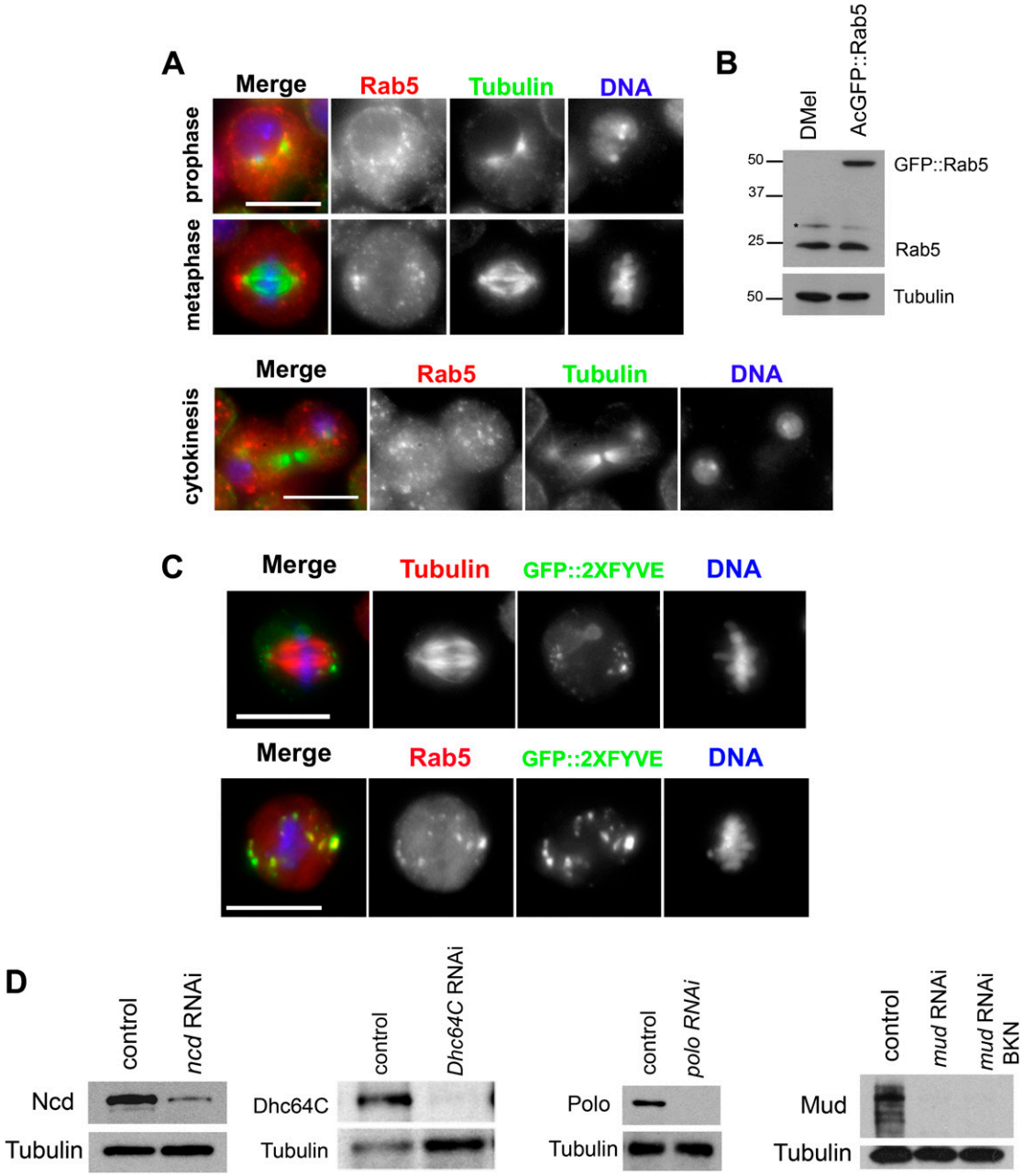


Fig. S2. (*A*) Rab5 localization. Cells were fixed and stained to reveal endogenous Rab5 (red), tubulin (green), and DNA (blue). (*B*) Western blot analysis of GFP::Rab5 and endogenous Rab5 expression. Tubulin was used as loading control. Expression levels of GFP::Rab5 and endogenous Rab5 were normalized against tubulin, and the ratio between GFP::Rab5/endogenous Rab5 was 0.8. The asterisk indicates unknown protein bands. (*C*) Cells were stained to detect an endosomal marker GFP::2XFYVE (green), DNA (blue), and either α-tubulin or Rab5 (red) in metaphase. (Scale bars: 10 μm.) (*D*) Western blot analyses of protein depletion levels after RNAi knockdown of Ncd, Dhc64C, Polo, and Mud. In all experiments, cells were treated with dsRNAs against GFP (control) or each of the four proteins for 3 d. Tubulin was used as loading control. Two different dsRNAs (1 and 2) directed against Mud efficiently depleted all three Mud isoforms.

Bait	Protein identified	CG #	Maximum Score	N° of Peptides
Rab5	Clathrin heavy chain	CG9012	656	29
	Lamin	CG6944	124	25
	GDP dissociation inhibitor	CG4422	102	4
	Dynein heavy chain	CG7507	82	22
	Rab4	CG4921	79	1
	Mud	CG12047	62	24
	Shibire	CG18102	59	2
Rab5+GTPγS	Lamin	CG6944	741	68
	Dynein heavy chain	CG7507	407	70
	Rab4	CG4921	106	3
	GDP dissociation inhibitor	CG4422	89	4
	Mud	CG12047	71	40
	Synaptojanin	CG6562	64	4
	Shibire	CG18102	59	3
Lamin	His2A	CG31618	1617	43
	His2B	CG17949	1086	31
	Otefin	CG5581	489	10
	Mud	CG12047	192	7
	Rab5	CG3664	60	1

Fig. S3. Rab5 interacting proteins in vivo. Partial list of Rab5 and Lamin-interacting proteins identified by MS. Rab5 complex purification was carried out with or without the addition of the analog GTPγS. Lamin, Mud, and Rab5 are highlighted in red. The CG number, maximum Mascot score (score), and number of identified peptides (peptides) are listed for each protein. Proteins identified in several separate purifications using other different tagged bait proteins or the PtA tag alone were considered common contaminants and excluded from this list.

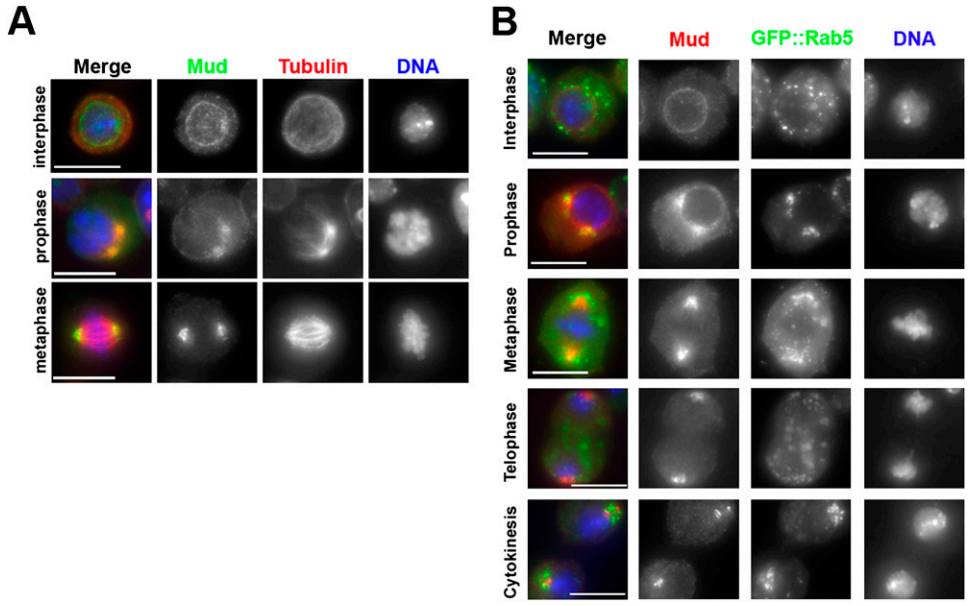


Fig. S4. GFP::Rab5 and Mud colocalize at prophase. (*A*) *Drosophila* S2 cells were fixed and stained to detect Mud (green), tubulin (red), and DNA (blue). (Scale bars: 10 μm.) (*B*) *Drosophila* S2 cells were fixed and stained to detect Mud (red), GFP::Rab5 (green), and DNA (blue). (Scale bars: 10 μm.)

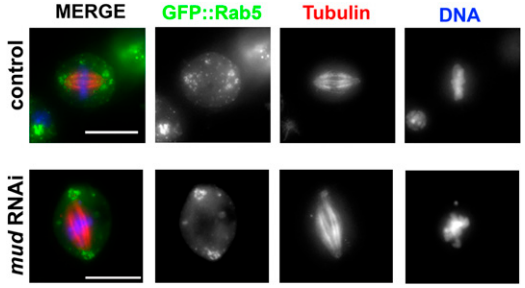
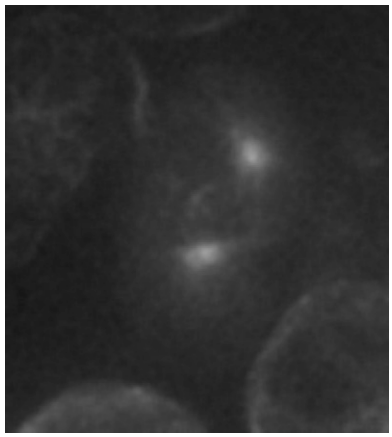
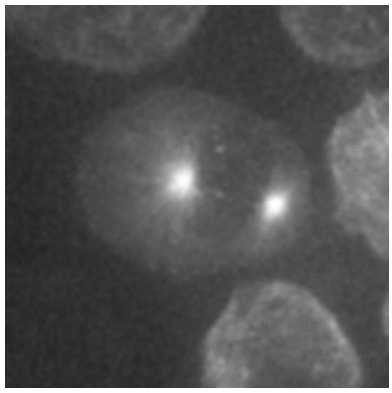


Fig. S5. Accumulation of GFP::Rab5 at the spindle poles in *mud* RNAi cells. *Drosophila* S2 cells expressing GFP::Rab5 were fixed and stained to reveal GFP (green), tubulin (red), and DNA (blue). (Scale bars: 10 μm.)



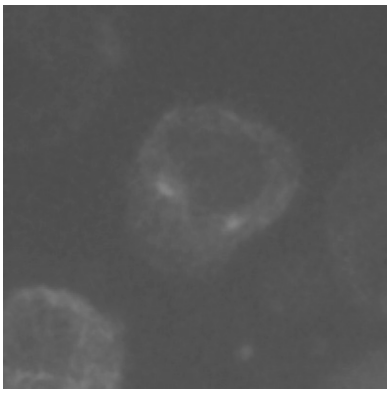
Movie S1. Polo::GFP dynamics during cell division in control cells. This movie shows Polo::GFP dynamics during mitosis from prophase to cytokinesis. Details are in Fig. 1. Frames were captured at 30-s intervals. Playback is 7 frames/s.

Movie S1



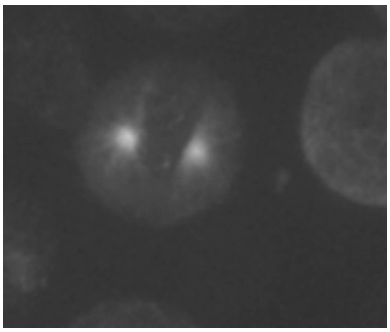
Movie S2. Polo::GFP dynamics in mitosis in an *Rab5* RNAi cell. Polo::GFP cells were treated with dsRNA directed against Rab5 for 3 d and then filmed from prophase. Details are in Fig. 1. Frames were captured at 1-min intervals. Playback rate is 7 frames/s.

Movie S2



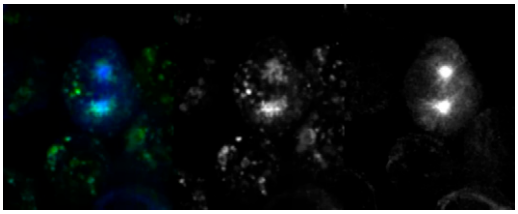
Movie S3. Polo::GFP dynamics in mitosis in an *Rab5* RNAi cell. Polo::GFP cells were treated with dsRNA directed against Rab5 for 3 d and then filmed from prophase. Details are in Fig. 1. Frames were captured at 1-min intervals. Playback rate is 10 frames/s.

Movie S3



Movie S4. Polo::GFP dynamics in mitosis in a *Rab5* RNAi cell. Polo::GFP cells were treated with dsRNA directed against Rab5 for 3 d and then filmed from prophase. This video shows cytokinesis defects after *Rab5* RNAi. Frames were captured at 1-min intervals. Playback rate is 7 frames/s.

Movie S4



Movie S5. Localization of GFP::Rab5 during mitosis. This movie shows GFP::Rab5 dynamics from prophase to cytokinesis. Frames were captured at 1-min intervals. Playback rate is 7 frames/s.

Movie S5