Appendix for

Centrosomal ALIX regulates mitotic spindle orientation by modulating astral microtubule dynamics


Table of contents

1. Supplementary Material and Methods
2. Supplementary References
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Antibodies and reagents

The following commercial antibodies were used at the indicated dilutions for Western blot (WB) and immunofluorescence (IF): rabbit anti-MAP1S (ab75503, 1:1000 WB and 1:100 IF), rabbit anti-Pericentrin (ab4448, 1:1000 IF), rabbit anti-NuMA (ab7585, 1:1000 WB), rabbit anti-Cep192 (ab122656, 1:500 WB, 1:100 IF), rabbit anti-Cep215/CDK5RAP2 (ab70213, 1:1000 WB, 1:100 IF), rabbit anti-GFP (ab6556 1:2000 WB), rat monoclonal anti-Deadpan (ab195173, 1:200 IF), rat anti-CLASP2 (ab95373, WB 1:1000) and goat anti-GST-HRP (ab58626, 1:1000 WB) were from Abcam. Mouse anti-α-tubulin (T5168, 1:20000 WB, 1:1000 IF), mouse anti-acetylated tubulin (T6793, 1:500 IF), rabbit anti-γ-tubulin (T3559, 1:1000 WB, 1:500 IF) and mouse anti-γ-tubulin (T6557, 1:500 IF) were obtained from Sigma Aldrich. Sheep anti-β/α-tubulin (ATN02, 1:200 IF) was from Cytoskeleton. Mouse anti-glutamylated tubulin (GT335, 1:500 IF) was obtained from Adipogen. Mouse anti-Cut (2B10, 1:200 IF) and mouse anti-FasIII (Mab7G10, 1:1000) were from DSHB. Rat anti-α-tubulin (mAb1864, 1:500) was bought from Serotec. Rabbit anti-aPKC (sc-10800, 1:100 IF) was from Santa Cruz. Rabbit anti-GCP3 (15719-1-AP, 1:1000 WB) was from Proteintech. Guinea pig anti-ALIX (1:1000 WB, 1:100 IF), rabbit anti-ALIX (1:5000 WB), rabbit anti-CHMP4B (1:1000 WB) and rabbit anti-Hrs (1:5000 WB) were generated as previously described (Cabezas et al, 2005; Eikenes et al, 2015; Malerod et al, 2007; Raiborg et al, 2001). Rabbit anti-Bazooka (1:100 IF) was provided by Tony Harris (University of Toronto, Canada) (Siller et al, 2006), guinea-pig anti-Bazooka (1:500 IF) and rabbit anti-Miranda (1:500 IF) were from Chris Doe (University of Oregon, OR) (Rolls & Doe, 2004), rabbit anti-Cnn (1:500 IF) was from Thomas C. Kaufman (Indiana University, IN) (Heuer et al, 1995), rabbit anti-Dgrip91 (1:100 IF) was obtained from Yixian Zheng (Harvard Medical School, Boston, MA) (Oegema et al, 1999), rabbit anti-Asl was from Caytano Gonzàlez (Institute for Research in Biomedicine Barcelona, Spain), rabbit anti-PON (1:100 IF) was provided by Yuh Nung Jan (Howard Hughes Medical Institute, UCSF) (Lu et al, 1998) and rabbit anti-Brat (1:100 IF) was from Jurgen A. Knoblich, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria) (Betschinger et al, 2006). All secondary antibodies used for confocal microscopy were purchased.
from Jackson ImmunoResearch Laboratories, whereas the secondary antibodies used for Western blot analysis were from LI-COR® Biosciences GmbH or Jackson ImmunoResearch Laboratories. Hoechst 33342 and Rhodamine-phalloidin were obtained from Life Technologies. Complete EDTA-free Protease Inhibitor Cocktail was from Roche. Laemmli loading buffer was from Bio-Rad. N-ethylmaleimide, Phosphatase Inhibitor Cocktail 2, and Thymidine were from Sigma Aldrich. All other chemicals were of analytical grade.

**Drosophila stocks and husbandry**

All fly stocks were maintained and fly crosses performed at 25°C. The previously characterized ALIX LOF fly strains, alix$^l$ (PBac(WH) ALiXf03094 from Exelixis at Harvard Medical School) and alix$^3$, as well as the FRT82B, alix$^l$-strain, were used in the current study (Eikenes et al, 2015). The genomic alix-l rescue line, containing the sequence of chromosome arm 3R spanning from 23500943 to 23586504, as well as the alix-l/CyO; alix$^l$/TM6B,Tb and alix-l/CyO; alix$^3$/TM6B,Tb stocks, were generated as previously described (Eikenes et al, 2015). The alix$^l$ flies were crossed with w$^{1118}$;Df(3R)BSC739/TM6C,Sb from Bloomington Drosophila Stock Centre (BDSC, Indiana University) to generate Df(3R)BSC739/alix$^l$ offspring. The w$^{1118}$ strain (3605) was used as wild type control, cnn$^{HK21}$ bw$^l$/CyO, l(2)DTS513$^l$ (cnn$^{HK21}$, 5039), w$^{1118}$;PBac(PSas-6$^{c0290}$) (Sas-6$^{c0290}$, 11148), y$^l$v$^l$/P(UAS-shRNAi-alix,chr3, TRiP.HMJO3123)attP40 (TRiP-alix RNAi, 33417) and y$^l$v$^l$/attP2 (TRiP control RNAi, 36303) were all purchased from BDSC (Indiana University). The Asl-YFP(T2), α-tubulin-GFP and γ-Tub23C$^{pl}$ flies (Sunkel et al, 1995) were provided by Caytano González (Institute for Research in Biomedicine Barcelona, Spain). The DSpd-2$^{G02143}$ stock was obtained from Jordan Raff (Sir William Dunn School of Pathology, Oxford, UK) (Dix & Raff, 2007)). The hsFlp, tubulin-GAL4, UAS-GFP;FRT82B, tubulin-GAL80/TM6B, Tb (MARCM82) strain was provided by Mark Peifer (University of North Carolina at Chapel Hill, NC).

**Immunofluorescence analyses of SOPs and mGSCs**

Clones of SOPs homozygote mutant for alix$^l$ mosaic analysis were induced by subjecting L3 larvae at a one hour heat shock at 37°C (genotype of the pupae: hs-Flp; FRT82B, alix$^l$/FRT82B, nls GFP). For indirect immunofluorescence, nota from
staged pupae were dissected in 1XPBS (BioWhittaker, Lonza, Belgium) and fixed in 4% paraformaldehyde-1XPBS (pH=7.2) for 15 minutes at room temperature. Primary antibodies against Cut and α-tubulin were incubated over night at 4°C. Fixed specimens were acquired using an LSM Leica SPE microscope equipped with a 63X plan Apo-N.A. 1.4. Testes of Drosophila males were dissected in PBS, fixed using 4% formaldehyde (30 minutes, room temperature) and stained with primary antibodies over night (4°C) as previously described (Haglund et al, 2010). Confocal fluorescence microscopy was then performed using Zeiss LSM780 equipped with a PlanApo 63x/1.40 oil DIC M27 objective.

**RNAi-mediated silencing of ALIX in NBs**

Knockdown in NBs was performed by crossing virgins of the NB driver line UAS-Dicer-2;insc-Gal4, UAS-CD8::GFP (kindly provided by Jürgen A. Knoblich, Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna) (Neumuller et al, 2011) to TRiP control (y¹v¹;attP2) or TRiP-alix RNAi males. Brains of larvae in the food were dissected four days after egg lay, fixed (4% formaldehyde in PBS-0.1% Triton X-100 for 20 min at room temperature), stained with primary antibodies over night (4°C) and examined by confocal fluorescence microscopy (Zeiss LSM 780).

**Cell lines and transfection**

HeLa cells were grown in DMEM (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Medium A). Caco-2 cells were cultured in DMEM (Gibco) supplemented with 15% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Medium B). All the cell lines were cultured in an atmosphere containing 5% CO₂ at 37°C according to ATCC. For knockdown analysis 3x10⁵ HeLa cells were transfected with indicated siRNA concentrations using RNAiMax transfection reagent according to the manufacturer (Life Technologies). Control siRNA (SI04380467, 25 nM), NuMA siRNA (SI04288116, 25 nM), γ-tubulin siRNA (SI02781142, 25 nM) and MAP1S siRNA (SI04236309, 25 nM) were obtained from Qiagen, whereas ALIX siRNA (D-004233-06, 25 nM) and ON-TARGETplus control siRNA (D-001810-01,
25 nM) were from Dharmacon. For double knockdown experiments the cells were transfected with (25 nM control siRNA + 25 nM ON-TARGET plus control siRNA), (25 nM ALIX siRNA + 25 nM control siRNA), (25 nM γ-tubulin siRNA/25 nM MAP1S siRNA + 25 nM ALIX siRNA) or (25 nM ON-TARGET plus control siRNA + 25 nM γ-tubulin siRNA/MAP1S siRNA). For the above siRNA treatments, cells were used and harvested 48 hours after transfection. When the cells were treated with control siRNA, ALIX siRNA, Hrs siRNA or CHMP4B siRNA the following siRNAs were used: Control siRNA (D-001810-01), ALIX siRNA (D-004233-06), Hrs siRNA (AS0224S1, Ambion) and CHMP4B siRNA (AS022M1, Ambion). The cells were transfected the first day with 50 nM control siRNA or treated with 50 nM Hrs or CHMP4B siRNA and then retransfected after 48 hours with 50 nM control siRNA, 25 nM control siRNA + 25 nM ALIX siRNA, 50 nM Hrs or CHMP4B siRNA, respectively. Cells were harvested 48 hours later, giving a total of 48 hours knockdown for ALIX and 96 hours knockdown for Hrs and CHMP4B. For the double transfection experiments shown in Figure 8G, H HeLa cells were first transfected with control or ALIX siRNA (25 nM) using RNAiMax and 4 hours later transiently transfected with pEYFP-C1 (0.25 µg), pEYFP-MAP1S (1 µg) (plasmids provided by Oliver J. Gruss, Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), Germany) (Tegha-Dunghu et al, 2014), pEGFP-C1 (0.25 µg) or pEGFP-CLASP2α (1µg) (provided by Anna Akhmanova, Faculty of Science, Utrecht University, The Netherlands) using the FuGene6 transfection reagent as suggested by the manufacturer (Promega). The cells were harvested 48h after transfection. Incubating the coverslips in 0.01 mM NaOH (pH=9.0) after the final rinse after secondary antibody incubation was performed to restore the GFP fluorescence of the quenched GFP signal caused by the low pH of the formaldehyde fixation (Katheder et al, 2017). Caco-2-organoids were generated as previously described with minor adjustments (Ivanov et al, 2008). RNAi experiments performed in Caco-2 cells followed a similar procedure as described above. Specifically, 7x10^5 cells were transfected with 25 nM control siRNA, ALIX siRNA (D-004233-06, Dharmacon), ALIX siRNA#2 (D-0042233-01, Dharmacon), ALIX siRNA#3 (D-0042233-04) or NuMA siRNA (SI04356940, Qiagen) using RNAiMax (Life Technologies). The following day, the Caco-2 cells were trypsinized and resuspended in 25 mM HEPES containing 15 % FCS. A cell:matrix mix was prepared of 1x10^6 cells, 10 mM HEPES,
2 mg/ml Collagen I (Trevigen) and 40% BD Matrigel® Matrix Basement Membrane Growth Factor Reduced (Corning) was cultured in LabTek-plates and allowed to solidify for 30 minutes (37°C). Medium B was applied to overlay the matrix, and renewed every second day until the Caco-2 cells had generated organoids (5-6 days after seeding) (Zhang et al, 2003).

**Immunostaining of human cells and Caco-2 cysts**

HeLa cells grown on coverslips were fixed in 4% formaldehyde (30 minutes, room temperature) or methanol (-20°C, 10 minutes), before they were permeabilized with PEM buffer [80 mM PIPES, 5 mM MgCl$_2$ x 6H$_2$O, pH=6.8] containing 0.05% saponin for 5 minutes (room temperature) and stained with primary antibodies (4°C, overnight). The coverslips were washed three times with PBS-0.05% saponin and stained with secondary antibodies for 1 hour at room temperature. After washing, the coverslips were mounted in Mowiol containing 1 µg/ml Hoechst 33342 for nuclear staining. Organoids of Caco-2 cells cultured in Matrigel were fixed (4 % formaldehyde, 1 hour, room temperature, slow rocking), washed three times (10 minutes per wash) with 1X IF wash buffer (pH=7.4) (diluted from 10X stock: 1.3 M NaCl, 105 mM Na$_2$HPO$_4$, 30 mM NaH$_2$PO$_4$, 77 mM NaN$_3$, 1% [wt/vol] BSA, 2% [vol/vol] Triton X-100, 0.4% [vol/vol] Tween-20) and incubated with primary antibodies (4°C overnight). The next day the cysts were rinsed three times in 1X IF wash buffer (20 minutes), incubated with secondary antibodies containing 1 µg/ml Hoechst 33342 for 2-4 hours, before they were washed three times in 1X IF wash buffer (20 minutes per wash). The specimens were next post-fixed using 4% formaldehyde (30 minutes, room temperature) and finally overlaid with PBS to prevent desiccation.

**Confocal immunofluorescence microscopy and imaging**

The specimens were examined using an LSM710 or LSM780 Zeiss confocal microscope with a PlanApo 63x/1.40 oil DIC M27 objective (if nothing else is stated). Images were acquired using the same settings below saturation when they were used for quantifications. The intensities of the different immunostainings were calculated by the ImageJ software. Image processing and preparation of the figures was made using ImageJ and Adobe Photoshop C4.
Live cell imaging of HeLa cells

HeLa Kyoto cells stably expressing GFP-tubulin and mCherry-phosho Histone-2B (HeLaK-GFP-tub-mCh-H2B) were grown in DMEM (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were transfected with control or ALIX siRNA using RNAiMax (Life Technologies) and the double-Thymidine block (2.5 mM Thymidine 17 hours- 8 hours release- 2.5mM Thymidine 17 hours- 6 hours release) started approximately two hours after transfection. A DeltaVision microscope equipped with Elite TruLight Illumination System equipped with 40x oil objective (Applied Precision) was used for the live cell imaging and images was captured in 10 minutes intervals. The structure of the mCherry-H2B stained chromatin and the mitotic spindle (visualized by GFP-tubulin) was used to characterize the different stages of cell division. Specifically we timed prometaphase from the onset of chromatin condensation to formation of the metaphase plane, and then metaphase was determined subsequently until separation of the chromatids (i.e anaphase onset). Anaphase was timed until the start of cleavage furrow ingression, which defines telophase onset. The time interval of telophase was measured until the start of chromosome decondensation (the two daughter cells are then connected with a thin intercellular bridge). Cytokinesis time was here subsequently measured from this point until bridge abscission.

Measuring mitotic spindle tilt in HeLa cells

Symmetrically dividing HeLa cells orient the spindle parallel to the substrate, implying that the centrosomes are ideally located in focus in the same confocal z-plane. Therefore, the spindle tilting was measured as the number of z-planes separating the localization of the two centrosomes. For each sample, z-stacks with depths of 0.32 µm per step were collected.

MT regrowth assay

Brains from wild type (w1118) and alix′ third instar larvae in the food (4 days after egg lay) were dissected in Schneider’s medium and incubated in cold Schneider’s medium on ice for 40 minutes to depolymerize the MTs. Regrowth of MTs was induced when
the brains were incubated in room-tempered Schneider’s medium for 0, 30, 60 or 300 seconds. The brains were fixed and permeabilized (4% formaldehyde in PBS-0.01% Triton X-100 20 minutes, room temperature) and stained with anti-α-tubulin and anti-Bazooka at 4°C overnight. HeLa cells depleted or not of ALIX, MAP1S or both were grown on coverslips and incubated on ice in cold medium for 30 minutes to depolymerize MTs. Regrowth of MTs was stimulated by incubating the cells at 37°C for 0, 60 or 420 seconds, before the cells were fixed (4% formaldehyde, 30 minutes at room temperature), permeabilized (0.05% saponin in PEM buffer for 5 minutes at room temperature), and stained with anti-α-tubulin, anti-Pericentrin and Hoechst 33342. Z-stacks of HeLa cells or NBs were acquired by the Zeiss LSM780 confocal microscope. Regrowth of MTs was assessed as the total fluorescence intensity of immunolabeled tubulin calculated from z-projections of single cells, related to control specimens (0 seconds regrowth) of the respective genotypes.

**Fractionation of polymerized and unpolymerized MTs**

HeLa cells transfected with control or ALIX siRNA were fractionated as previously described (Srivastava & Chakrabarti, 2014). Briefly, the cells were rinsed in PBS and lysed in MT lysis buffer ([50 mM HEPES, pH 7.4, 150 mM NaCl, 1% [vol/vol] NP-40, 0.5% sodium deoxycholate, 0.1% [vol/vol] SDS) containing Complete protease inhibitor cocktail. Cell debris and nuclei were removed by centrifugation (20,800 g, 10 minutes, 4°C) before the post-nuclear supernatant was further fractionated by ultracentrifugation at 244,413 g for 1 h (rotor TLA 120.1, Beckman Coulter). The generated pellet of polymerized MTs (P) and the corresponding supernatant of non-polymerized tubulin (S) were collected and used for SDS-PAGE.

**Western blotting analysis**

Human cells were rinsed with PBS and lysed in [25mM HEPES (pH=7.2), 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 0.5% NP-40, 1 mM DTT and Complete protease inhibitor cocktail] on ice for 30 minutes. *Drosophila* tissue was lysed in HEPES lysis buffer [50 mM Tris (pH=8.0), 150 mM NaCl, 50mM HEPES, 1 mM EGTA, 1 mM EGTA, 25mM NaF, 0.5% NP-40, 1 % Triton X-100, 10 μM ZnCl₂] supplemented with Complete protease inhibitor cocktail. Nuclei and cell
debris were cleared by spinning the samples at 20,800g for 10 minutes (4°C). Equal amounts of proteins were denatured by boiling in Laemmli buffer containing 50 mM DTT, separated by SDS-PAGE and blotted onto PVDF membranes (Millipore Corporation). The membranes were blocked by air-drying, re-wet in PBS-0.01% Tween, incubated with primary antibodies (4°C overnight: ALIX, Cep192, Cep215, MAP1S, NuMA, CLASP2, GCP3, GFP, Hrs and CHMP4B) or 1 hour at room temperature: γ-tubulin, α-tubulin, GAPDH, GST-HRP), washed with PBS-0.01% Tween three times, incubated with fluorescently labeled secondary antibodies (LI-COR® Biosciences GmbH) for 1 hour at room temperature and rinsed with PBS-0.01% Tween twice and finally PBS. The membranes were scanned using the Odyssey developer and the intensities of the immunoreactive bands were quantified by the Odyssey software (LI-COR® Biosciences GmbH). When HRP-conjugated antibodies (Jackson ImmunoResearch Laboratories) were used for detection of Hrs, CHMP4B, ALIX and CLASP2, the membranes were additionally blocked for 1h with 5% BSA before incubation with primary antibodies. The membranes were developed using SuperSignal West Dura Extended Duration substrate (Thermo Scientific) and ChemiDoc Imagers (BioRad).

**GST pull-down**

Larvae (30) were lysed in HEPES lysis buffer supplemented with fresh protease (Sigma-Aldrich) and phosphatase (PhosSTOP, Roche Diagnostics) inhibitors. The post-nuclear supernatant was divided in two and incubated with GST or GST-ALIX conjugated to sepharose beads (rotating at 4°C, over night). The beads were rinsed three times in HEPES lysis buffer, boiled in Laemmli buffer (with 0.1 M DTT) before the eluted proteins were subjected to SDS-PAGE in order to detect Dgrip91, γ-tubulin and GST.

**In vitro binding assay**

The Drosophila alix cDNA was subcloned from the pENTR1A-alix previously described (Eikenes et al, 2015) into pDEST15. The empty pDEST15 vector served as negative control. The constructs were transformed into BL21 E.coli and the IPTG-induced proteins were purified using glutathione-sepharose according to the manufacturer (GE Healthcare). T7-promoter-tagged PCR-products of γ-tubulin and
Dgrip91 were amplified from a Drosophila cDNA library (Drosophila Genomics Resource Center), whereas DSpd-2 and Cnn were amplified from pDONR_Zeo_Spd-2 1-1146 and pDONR_Zeo_Cnn-plasmids kindly provided by Jordan Raff (Sir William Dunn School of Pathology, Oxford, UK). The following primers were used:

- T7-\(\gamma\)-tub23C-forw: 5'-TAATACGACTCACTATA\(\gamma\)GAGAATGCCAAGTGAAATAATTACTTT
- \(\gamma\)-tub23C-rev: 5'-CTAGGAACCGGCCGCTGTCACAGAT
- T7-Dgrip91-forw: 5'-TAATACGACTCACTATAGGGAGAATGTGCAGGACAGGAT
- Dgrip91-rev: 5'-CTATTCCTGAGTGGACGGCGTACATATCACGAAG
- T7-Cnn-forw: 5'-TAATACGACTCACTATAGGGAGAATGAATAGTAATCGAACGTCGT
- Cnn-rev: 5'-TTATAACTCATTCTCCATGTTTGAGCGAACGTTTCG
- T7-DSpd-2-forw: 5'-TAATACGACTCACTATAGGGAGAATGGACAGTAGCAGTGGAAG
- DSpd-2-rev: 5'-TAAAATTTAAAAACTAATCGGGACACTGATGCGCTCGTT

Since the forward-primers harbored a T7 promoter-sequence, the generated PCR-products (1 \(\mu\)g) were used for in vitro reticulocyte-mediated translation in the presence of \(\textsuperscript{35}\)S-methionine (TNT\textsuperscript{\textregistered} T7 Quick Coupled Transcription/Translation System, Promega). For the in vitro binding assay bacterially expressed GST or GST-ALIX conjugated to sepharose-beads was incubated with \(\textsuperscript{35}\)S-labelled-\(\gamma\)-tubulin, Dgrip91, DSpd-2 or Cnn in NET-N buffer ([20 mM Tris-HCl (pH=8.0), 100 mM NaCl, 6 mM EDTA, 0.5% [vol/vol] NP-40]) containing Complete protease inhibitor cocktail. After 1 hour rotation (4°C), the samples were washed five times with NET-N buffer, before the beads were resuspended in Laemmli buffer with 50 mM DTT and subjected to SDS-PAGE. The membrane was dried and subjected to autoradiography using a PharosFX scanner to detect interaction between GST-ALIX and \(\textsuperscript{35}\)S-labelled proteins. The amount of GST and GST-ALIX were visualized by Ponceau S-staining the membrane.

**Mass spectrometry**

The protein samples were separated by SDS-PAGE, the gel was silver stained and whole lanes were subjected to protein identification, as previously described (Lorey et al, 2017). Briefly, each lane was cut into five pieces, the proteins were in-gel digested into peptides with trypsin (Promega) and the resulting peptides were desalted and concentrated before mass spectrometry by the STAGE-TIP method using a C18 resin.
Each peptide mixture was analyzed by a nEASY-LC coupled to QExactive (ThermoElectron, Bremen, Germany) with EASY Spray PepMap®RSLC column (C18). The resulting MS raw files were submitted for protein identification using Proteome Discoverer 2.1 (ThermoFisher) and Mascot 2.4 (MatrixScience) search engine. The search criteria for Mascot searches were: trypsin digestion with one missed cleavage allowed, Carbamidomethyl (C) as fixed modification and Acetyl (N-term), Gln->pyro-Glu (N-term Q), Oxidation (M) as dynamic modifications. The parent mass tolerance was 5 ppm and MS/MS tolerance 0.05 Da. The SwissProt database was used for the database searches. All of the reported protein identifications were statistically significant (p<0.05) in Mascot.

GFP-trap immunoprecipitation

For GFP trap precipitation experiments 3x10⁵ HeLa cells (per well in a 6-well plate) were seeded and transfected with either 1 µg pEYFP or 1 µg pEYFP-MAP1S-full length (FL), 1 µg pEYFP-MAP1S-heavy chain (HC), 1 µg pEYFP-MAP1S-light chain (LC) using the FuGENE® 6 Transfection reagent as described by the manufacturer (Promega). The cells were synchronized in mitosis by double thymidine block (16 hours 2.5 mM thymidine, 8 hours release in medium A, 16 hours 2.5 mM thymidine and 1 hour release in medium A). Specifically, transfected cells from a 6-well plate (per transfection condition) were pooled and lysed (GFP trap lysis buffer [10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40] containing Complete protease inhibitor cocktail, Phosphatase Inhibitor cocktail 2 and 5 mM N-Ethylmaleimide) before cell debris and nuclei were removed by centrifugation (20,800×g, 7 minutes, 4°C). The post-nuclear supernatant was diluted with Washing buffer ([10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA] with protease inhibitor) and incubated with 15 µl GFP beads for 1 hour (4°C). Beads with bound proteins were washed (3 times) before the precipitated proteins were eluted by boiling (10 minutes) in Laemmli sample buffer (Bio-Rad) containing 50 mM DTT. The eluted proteins were subjected to SDS-PAGE and immunoblotted with antibodies against GFP and ALIX.
**Statistical analysis**

The nested ANOVA-test was used for the statistical analysis of the variation in the mitotic spindle orientation between wild type and *alix*-mutant cells. ANOVA with Dunnet's adjustment for multiple testing was used for the statistical analyses of the variation in the mitotic spindle orientation or tilt between control and ALIX siRNA Caco-2 cells and control, ALIX, Hrs and CHMP4B siRNA HeLa cells, respectively. Comparison of fluorescence intensities, number of mGSCs with abnormal centrosome location and duration of cell cycle-phases was performed using paired, two-tailed Student's t-test. For both tests, *p*-values below 0.05 were regarded as significantly different results.

**Supplementary References**


Rolls MM, Doe CQ (2004) Baz, Par-6 and aPKC are not required for axon or dendrite specification in Drosophila. *Nature neuroscience* 7: 1293-1295


