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Supplemental Information

Tiam1-Rac Signaling Counteracts Eg5 during Bipolar Spindle Assembly to Facilitate Chromosome Congression

Simon A. Woodcock, Helen J. Rushton, Eduardo Castañeda-Saucedo, Kevin Myant, Gavin R.M. White, Karen Blyth, Owen J. Sansom, and Angeliki Malliri

Figure S1 (related to Figure 1)

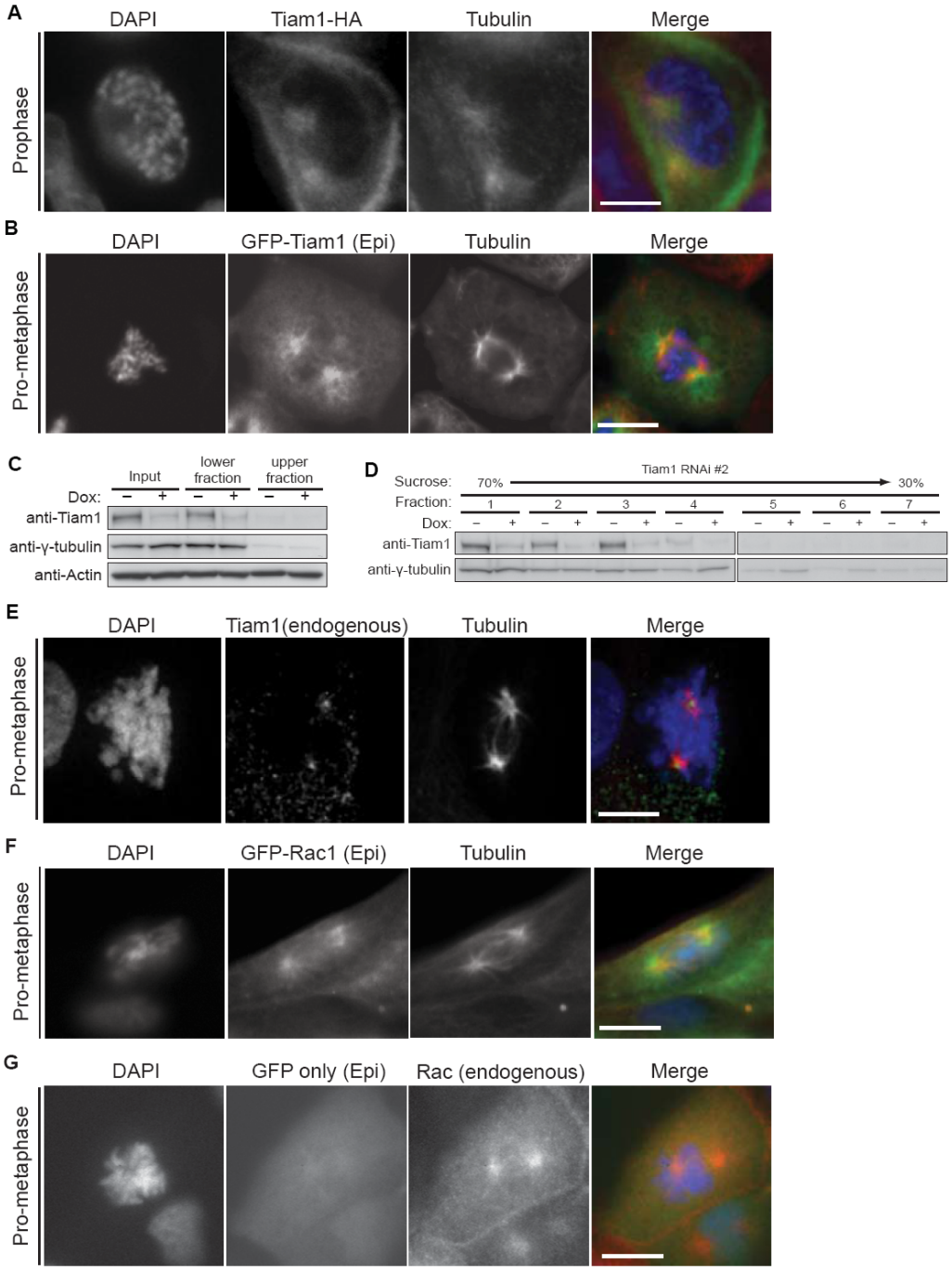

Figure S1. Tiam1 and Rac are centrosome-associated proteins

(**A**) MDCK II cells stably expressing HA-tagged Tiam1 were fixed and co-stained with anti-HA (green), anti-β-Tubulin (red) and DAPI (blue). A prophase cell is shown. Scale bar, 10 μm. (**B**) MDCK II cells stably expressing Tiam1-GFP were

fixed and stained with anti-α-Tubulin (red) and DAPI (blue). Tiam1 localisation was detected using epifluorescence from the GFP tag. A pro-metaphase cell is shown. Scale bar, 15 μm. (**C**) A crude centrosome purification from control or Tiam1-depleted MDCK II cells (RNAi #2; plus dox), was prepared as described in Supplemental Experimental Procedures. Levels of Tiam1, γ-tubulin and actin in the input, and the upper and lower fractions of the crude centrosome preparation, were determined by immunoblotting. (**D**) The preparations from panel **C** were further purified over a discontinuous sucrose gradient as described in Supplemental Experimental Procedures. Tiam1 and the centrosomal protein γ-tubulin were detected in the resulting fractions by immunoblotting. (**E**) MDCK II cells were fixed and stained with anti-Tiam1 (green), anti-tubulin (red), and DAPI (blue). A single z plane at the level of MT asters is shown from a pro-metaphase cell. Scale bar, 5 μm. (**F**-**G**) MDCK II cells stably expressing GFP-Rac1 (**F**) or a GFP-tag only (**G**) were fixed and stained where indicated with anti-tubulin or anti-Rac (red) and DAPI (blue). The GFP tag (green) was detected using epifluorescence. Cells in pro-metaphase are shown. Scale bars, 15 μm.

Figure S2 (related to Figure 2)

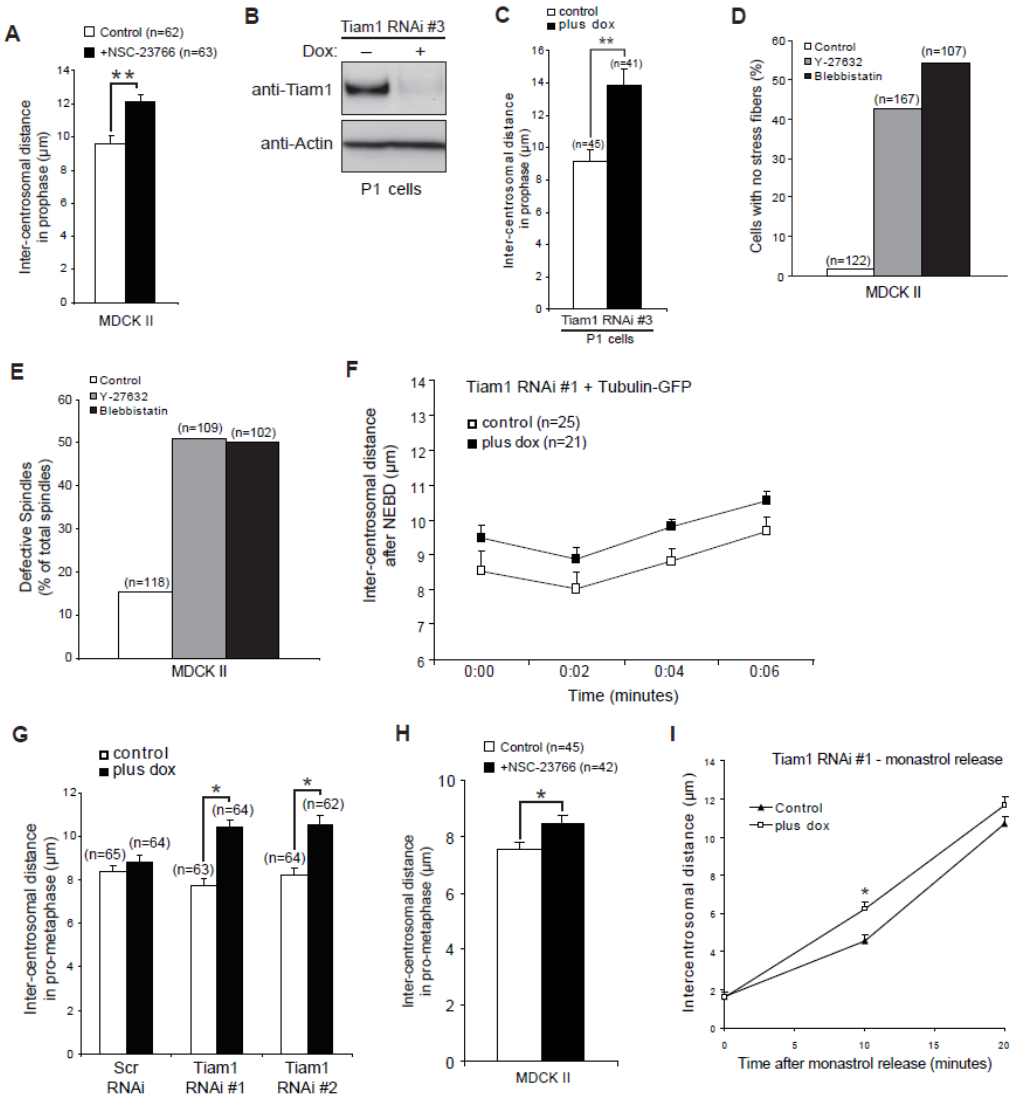

Figure S2. Tiam1-Rac signalling antagonises centrosome separation in early mitosis

(**A**) Control MDCK II cells treated with the Rac inhibitor NSC-23766 or left untreated were fixed and co-stained with anti-α-tubulin, anti-γ-tubulin (or anti-pericentrin) and DAPI. The distance between centrosomes in prophase cells was measured in 3D. (**B-C**) P1 cells carrying a doxycycline-inducible system with shRNA targeted to Tiam1 (RNAi #3) were treated with doxycycline (dox) where indicated. (**B**) Lysates were prepared and levels of Tiam1 and actin protein were detected by immunoblotting. (**C**)

Cells were fixed and stained as in panel **A**. The distance between centrosomes in prophase cells was measured in 3D. (**D-E**) Control MDCK II cells treated with vehicle (DMSO), Y27632 (20μM for 25mins) or blebbistatin (50μM for 25mins), were fixed and co-stained for anti-α-tubulin, phalloidin and DAPI. Cells were scored for (**D**) the presence of actin stress fibers, and (**E**) defective mitotic spindles (as described previously [10]). (**F**) Control and Tiam1-depleted cells (RNAi #1; plus dox) expressing GFP-tagged β-tubulin were analysed using fluorescence time-lapse microscopy. The distance between centrosomes at each time-point after NEBD (i.e. pro-metaphase) was measured in 3D. (**G-H**) The inter-centrosomal distance was measured for the indicated pro-metaphase cells prepared as in panel **A**. (**I**) Control and Tiam1-depleted cells (RNAi #1; plus dox) were arrested with monopolar spindles in pro-metaphase using Monastrol (50μM for 4hrs) and either fixed, or released from the drug for 10 or 20mins to allow mitosis to proceed and fixed. Samples were stained as in panel **A**, and the distance between centrosomes in over 90 pro-metaphase cells was measured in 3D. In all cases data is presented as the mean +SEM. * $p < 0.05$, ** $p < 0.001$.

Figure S3 (related to Figure 3)

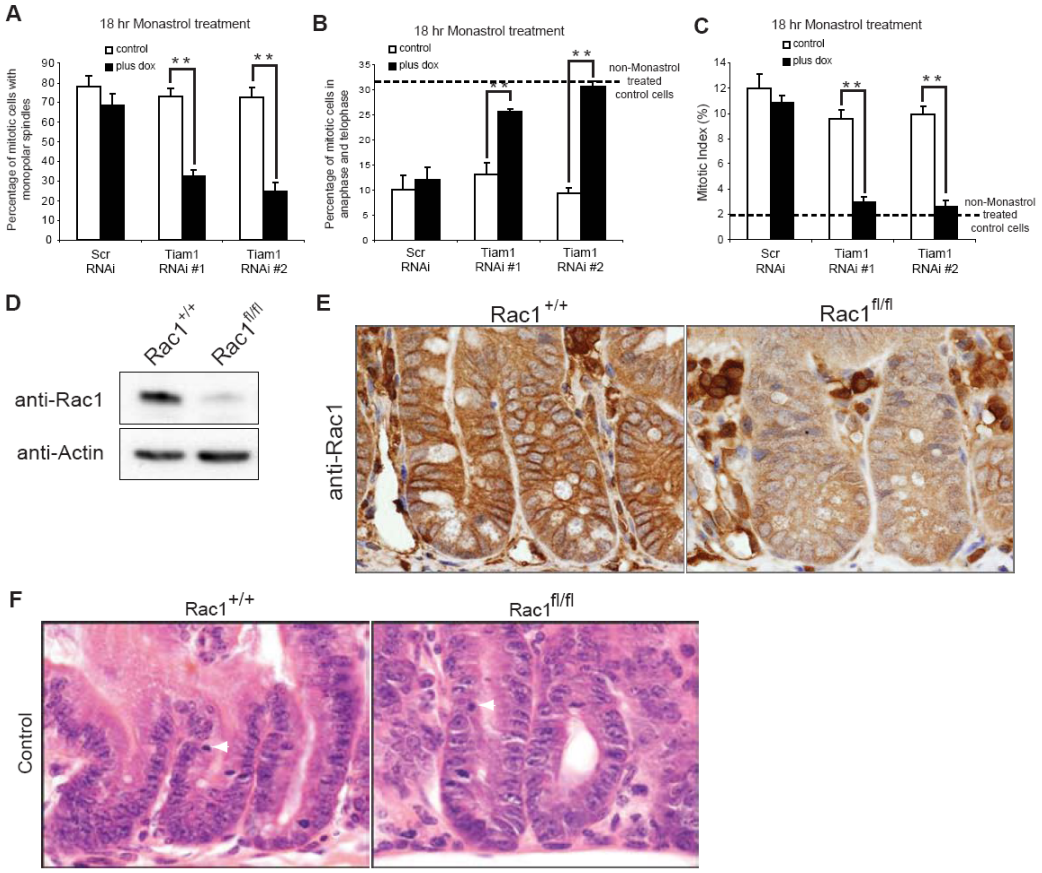

Figure S3. Tiam1-Rac signalling antagonise Eg5 *in vitro* and *in vivo*

(**A-C**) Inducible MDCK II shRNA cell lines, treated with dox where indicated, were treated with Monastrol (25μM for 18 hrs) and fixed. Fixed samples were co-stained with anti-α-tubulin, anti-γ-tubulin and DAPI. The fraction of mitotic cells with monopolar spindles (>120 mitotic cells) (**A**), the fraction of mitotic cells in anaphase and telophase (>120 cells) (**B**) or the mitotic index (from 550-1100 cells) (**C**) was determined by manual counting. The dotted lines (**B-C**) indicate the normal fraction of mitotic cells in anaphase and telophase (**B**), and the normal mitotic index (**C**), observed in Eg5-inhibitor-untreated control cells. Data presented as the mean of at least 3 separate experiments +SEM. ** $p < 0.01$. (**D-F**) *AhCre Rac1*$^{+/+}$ (*Rac1*$^{+/+}$) and *AhCre Rac1*$^{fl/fl}$ (*Rac1*$^{fl/fl}$) mice were treated with β-napthoflavone to induce Cre

expression. Three days post Cre induction levels of Rac1 protein in the intestinal epithelium were determined by immunoblot (**D**) and immunohistochemistry (**E**). (**F**) H&E staining of intestinal crypts three days post Cre induction from control (STLC-untreated) *Rac1*$^{+/+}$ and *Rac1*$^{fl/fl}$ mice. Example mitotic figures are highlighted with white arrowheads.

Figure S4 (related to Figure 4)

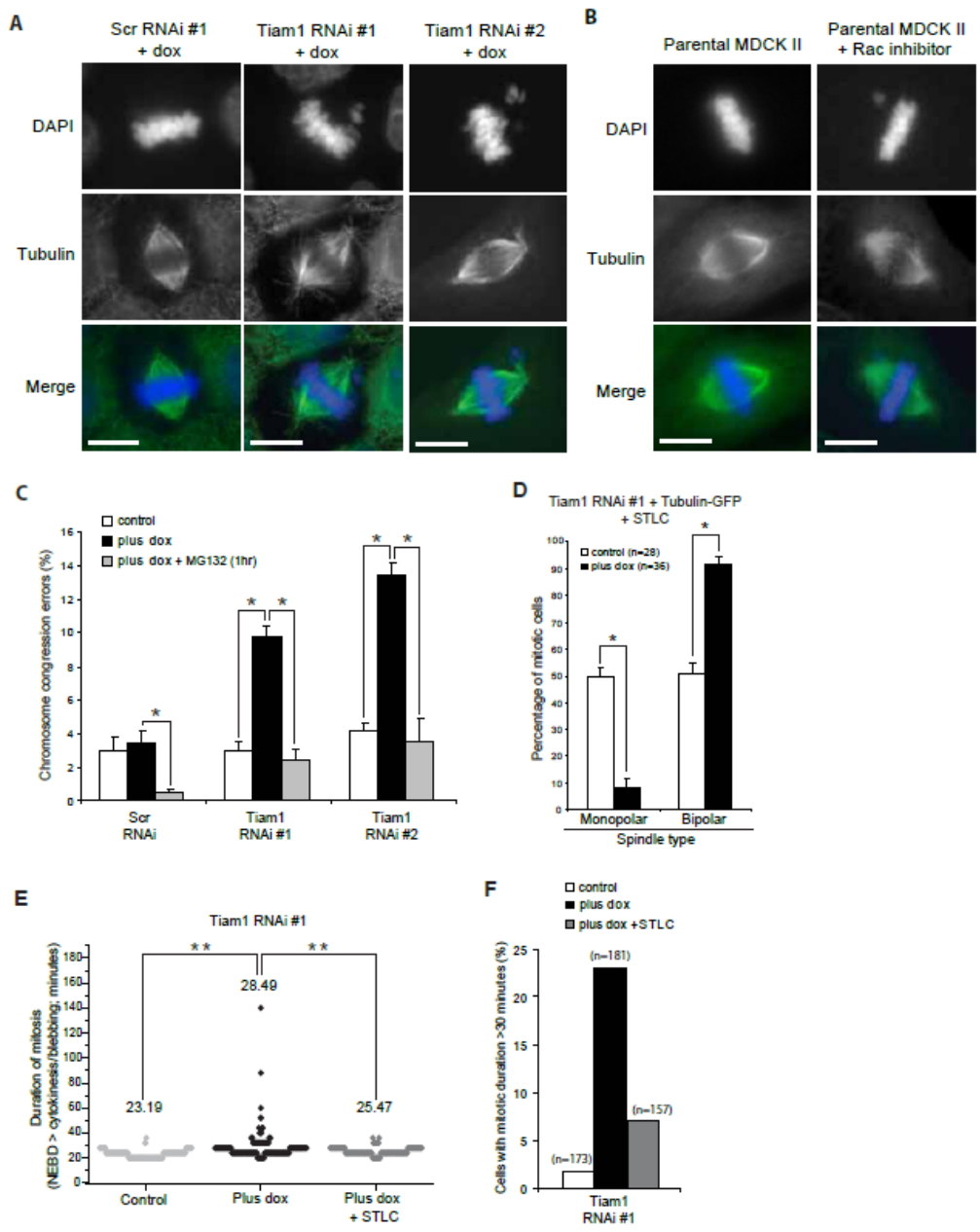

Figure S4. Tiam1-Rac signalling allows proper chromosome congression and mitotic progression through regulating centrosome separation

(**A**-**B**) Control or Tiam1-depleted (RNAi #1 and #2; plus dox) MDCK II cells (**A**) or parental MDCK II cells synchronised by double thymidine block (as described in Supplemental Experimental Procedures) and released into Rac inhibitor (NSC-23776

for 6-8hrs) or mock-treatment (**B**), were fixed and co-stained with anti-α-tubulin (green) and DAPI (blue). Example images show normal chromosome congression in control samples, and congression errors in Tiam1-depleted or Rac inhibitor treated cells. (**C**) To confirm that the increased proportion of cells with congression errors in Tiam1-depleted cells was due to slower chromosome congression (i.e. an increased pro-metaphase duration), we treated control and Tiam1-depleted (RNAi#1 and #2; plus dox) cells with MG132 (20μM for 1 hr - to arrest cells at the metaphase/anaphase transition) where indicated. Cells were fixed and stained as in panel **A**, and chromosome congression errors were determined as described in Supplemental Experimental Procedures (80-300 cells/rep). (**D**) Control and Tiam1-depleted (RNAi#1; plus dox) cells expressing GFP-tagged β-tubulin and treated with STLC were analysed using fluorescence time-lapse microscopy. The proportion of mitotic cells that formed monopolar versus bipolar spindles after NEBD was scored. Cells that formed monopolar spindles did not subsequently recover or complete mitosis and were therefore excluded from subsequent analysis. (**E**-**F**) The mitotic duration (calculated as minutes from the first frame after NEBD to the first frame with clear evidence of cytokinesis and/or membrane blebbing) of >150 cells was measured, using phase-contrast time-lapse microscopy, for control and Tiam1-depleted (RNAi #1; plus dox) cells treated with STLC where specified. All measurements are shown in panel **E**, with the mean duration shown above the data points, whilst panel **F** shows the proportion of cells displaying a mitotic duration over 30 minutes. Data presented as the mean of all measurements +SEM. * $p < 0.05$, ** $p < 0.001$. Scale bars, 10 μm.

Supplemental Experimental Procedures

Constructs

The production of the single-vector tetracycline-repressor based inducible shRNA system is described in detail below; the specific 'target' RNAi sequences were: RNAi #1; GAGGTTGCAGATCTGAGCA, RNAi #2; ATACGGCCAGTGATAATTA, RNAi #3; GCAAGTCTCTTGGGAGGAG, and Scrambled (Scr); ATGAAGTCGC ATGGTGCAG. Tiam1 carrying a C-terminal HA-tag in the retroviral LZRS-IRES-Zeo (where IRES is an internal ribosome entry site) expression vector was constructed previously [1], and was modified with a C-terminal GFP-tag. RNAi#1-resistant wild-type Tiam1 was cloned into the retroviral construct (Tiam1-HA-IRES-DsRed); GEF-mutant Tiam1 (Q1191A, K1195A) was generated via QuikChange site-directed mutagenesis (Stratagene). pGFP-Rac1^{WT} was a kind gift from F. Sanchez, pBOS-Histone-2B-GFP was a kind gift from G. Lacaud and pGFP-β-Tubulin was kindly donated by V. Allan.

Inducible shRNA system

Sense and antisense DNA oligonucleotides were synthesised by MWG; Sense: 5'-GATCCC**XXXXXXXXXXXXXXXXXXX***TTCAAGAGA***YYYYYYYYYYYYYYYYYYY**TTTTTGGAAA-3', Antisense: 5'-AGCTTTTCCAAAAA**XXXXXXXXXXXXXXXXXXX***TCTCTTGAA***YYYYYYYYYYYYYYYYYYY**GG-3'. These oligo-nucleotides contained the RNAi target sequences (denoted by '**X**') and the reverse complement of the RNAi target sequence (denoted by '**Y**'); italics indicate the 9 base pair hairpin loop. Sense and Antisense oligonucleotides were annealed in ligation buffer (Roche) by incubation for 2 minutes at 95 °C, 5 minutes at 50 °C before being

placed on ice. Annealing was confirmed by standard agarose gel electrophoresis. The H1 promoter/Tet-operator (TetO) fragment from pTER [2] was cloned into pGEM-T-easy (Invitrogen) flanked with *Xho*I sites. The resulting construct (*Xho*I-H1/TetO-*Xho*I in pGEM-T-easy) was digested with *Bgl*II and *Hind*III, and the annealed RNAi oligonucleotides were ligated. The resulting plasmid was digested with *Xho*I and the fragment containing the entire inducible shRNA sequence (*Xho*I-H1/TetO:shRNA-*Xho*I), was purified. This *Xho*I fragment was cloned into the single *Xho*I site of pN1pβactin-TetR-IRES-eGFP that in addition contained a tetracycline-repressor (TetR)-IRES-eGFP module under the control of the β-actin promoter (produced by replacing the sequence of rtTA2S-M2 in pN1pβactin-rtTA2S-M2-IRES-eGFP [3] with the sequence of TetR). This produced a single-vector inducible shRNA system, that following the addition of tetracycline or doxycycline (in order to relieve inhibition by the TetR) expresses the target shRNA; in addition the construct expresses a G418-resistance gene and GFP which can be used to isolate cells by FACS, to facilitate the production of stable cells lines [3]. A similar BFP-based single-vector inducible shRNA system was also created by replacing the sequence IRES-eGFP in pN1pβactin-TetR-IRES-eGFP with the sequence of IRES-BFP. This enables the isolation of stable inducible shRNA cell lines by FACS, whilst also allowing the introduction and detection of GFP-tagged proteins, such as β-tubulin-GFP, in the same cells.

Cell culture, transduction and treatment

MDCK II and P1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) in the presence of 10% foetal bovine serum (FBS; Gibco). Cell lines transduced with the inducible shRNA system were maintained in DMEM

supplemented with 10 % tetracycline-free FBS (Autogen Bioclear) and 1 mg/ml G418 (Sigma). All cells were cultured in a 37^{o}C, 5% CO_2 incubator. To generate stable shRNA cell lines, MDCK II cells were transfected with the single-vector inducible shRNA plasmids using Fugene 6 (Roche), selected with G418 for two weeks before BFP or GFP-positive cells were sorted using FACS. To generate stable pools expressing Histone-2B-GFP, β-Tubulin-GFP and Rac1-GFP, the Tiam1 RNAi#1 line (expressing BFP as a marker) was transfected with 1μg of pBOS-H2B-GFP or pGFP-β-Tubulin, and parental MDCK II was transfected with 1μg pGFP-Rac1^{WT}. H2B-GFP expressing cells were selected with Blasticidin (Invitrogen) for 2 weeks before FACS sorting a stable pool of cells. Rac-GFP and β-Tubulin-GFP cells were FACS-sorted for a GFP positive pool 4 days after transfection, then re-FACS-sorted 7 days later to obtain a stable pool. To generate MDCK II cells expressing GFP or HA-tagged Tiam1, LZRS-Tiam1-GFP-IRES-Zeo or LZRS-Tiam1-HA-IRES-Zeo were introduced into MDCK II cells by retroviral transduction as previously described [1] and a stable pool of cells selected with Zeocin (Invitrogen). To obtain cells expressing RNAi-resistant Tiam1, the WT and GEF-mutant Tiam1-HA-IRES-DsRed constructs were retrovirally transduced and DsRed-positive cells were selected for further analysis. For inducible shRNA experiments, cells were treated with doxycycline (dox) at a final concentration of 0.2 μg/ml (or the equivalent amount of vehicle control – ethanol) for a total of 3-4 days before analysis. For synchronisation with double thymidine block, cells were incubated with 2 mM thymidine for 16 hr, released into fresh medium for 6 hr, and incubated for a further 16 hr with 2mM thymidine before release into fresh medium for the indicated times. Unless stated otherwise, cells were incubated with Eg5 inhibitors Monastrol (25 μM, Sigma) or S-trityl-L-cysteine (STLC; 1 μM, Calbiochem) for the indicated times. Cells were treated with ROCK

inhibitor (Y27632; 20 μM, Sigma) or Myosin-II inhibitor (Blebbistatin; 50 μM, Sigma) for 25 minutes, with Rac inhibitor (NSC-23766; 100 μM, Calbiochem) for 6-8 hours, with proteasome inhibitor (MG132; 20 μM, Sigma) for 1 hour, or with MT stabiliser (Taxol; 120nM, Sigma) for 6 hours.

Mouse experiments

All experiments were according to UK Home office regulations. Mice were backcrossed to C57BL6J background for 10 generations and experiments were performed on mice at 6-8 weeks of age. Genotyping of the *AhCre* transgene and the *Rac1* floxed allele was performed as previously described [4, 5]. STLC was a kind gift from Frank Kozielski. It was dissolved in 10% DMSO and injected at 25mg/kg intraperitoneally (ip). To induce Cre mediated recombination, 3 ip injections of β-napthoflavone were given in a single day to *AhCre Rac1*$^{fl/fl}$ and *AhCre Rac1*$^{+/+}$ mice as previously described [6]. At the appropriate time-point mice were culled and the small intestine removed and flushed with water (at least 3 mice were used for each experiment) and fixed as previously described [6]. Immunohistochemistry for Rac1 was performed using mouse anti-Rac1 (MAB3735; 1:400, Upstate) following antigen retrieval using citrate buffer. For protein extraction, an epithelial extraction was performed as previously described [7]. Mitotic index and the percentage of mitotic cells in anaphase were scored from haematoxylin and eosin (H & E) stained sections as previously described [8]. For each analysis, 25 full crypts were scored from at least 3 mice of each genotype.

Immunoblot analysis

Analysis of protein levels in cell lysates was performed essentially as previously described [1]; using anti-Tiam1 (C16; 1:1000, Santa Cruz), anti-γ-tubulin (polyclonal; 1:1000, Sigma), anti-Rac1 (MAB3735; 1:1000, Upstate) and anti-β-Actin (Clone AC-15; 1:5000, Sigma) primary antibodies, and subsequently horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5,000, GE Healthcare) and visualised by enhanced chemiluminescence (Perkin Elmer).

Centrosome preparation

Centrosomes were prepared essentially as described previously [9]. Briefly, MDCK II cells were treated with 4μM Cytochalasin D (Sigma) and 600nM Nocodazole (Sigma) in DMEM for 3 hours before harvesting. All subsequent steps were performed on ice or at 4°C. Cells were washed successively in PBS, 0.1xPBS/8% sucrose and finally 8% sucrose, before being lysed (1mM Tris-pH 8.0, 0.5% triton, 0.1% β-mercaptoethanol, 0.5mM $MgCl_2$ and EDTA-free Complete protease inhibitor cocktail (Roche)). Lysate was cleared by 5 minutes centrifugation at 1,500g and passing through a 33μm filter, before supplementing with PIPES and EDTA to final concentrations of 10mM and 1mM respectively. The lysate was layered over a 60% sucrose (w/v) cushion [in PE (10mM PIPES-pH 7.0, 1mM EDTA) containing 0.1% triton] and centrifuged at 25,000g for 30 minutes to sediment centrosomes onto the cushion. The upper fraction was removed, whilst the remaining lower fraction and sucrose cushion, enriched for centrosomes, were thoroughly mixed [resulting in ~30% sucrose (w/v) solution] and loaded onto a discontinuous sucrose gradient [70%, 50%, and 40% (w/v) in PE containing 0.1% triton], and centrifuged at 100,000g for 1 hour. Fractions (200μl) were collected from the bottom; each fraction was diluted with 1ml

of PE buffer and centrosomes were recovered by centrifugation at 16,000g for 20 minutes. Supernatants were removed and the pelleted centrosomes re-suspended in SDS sample buffer and processed for SDS-PAGE as described above.

Immunofluorescence

Fixation for detection of endogenous Tiam1 at centrosomes was achieved by washing cells on glass coverslips briefly with 37°C PBS, followed by incubation in 3.7% formaldehyde/ 0.5% triton (in PBS, pre-warmed to 37°C) for 7.5 mins. After 2 rapid exchanges of PBS at room temperature (RT), cells were subsequently incubated in 3.7% formaldehyde (PBS, RT) for an additional 7.5 mins. All steps were performed at RT and, following washes, immunofluorescence staining was commenced immediately, with overnight incubation in the primary antibody (see below). For all other stainings cells were processed and stained essentially as previously described [1]. For immunofluorescence analysis, fixed cells were stained with the following primary antibodies: anti-Rac1 (Clone 102; 1:200, BD), anti-Tiam1 (1:100, Bethyl), anti-HA-Alexa Fluor 594 conjugate (Clone 16B12; 1:500, Molecular Probes), for detection of microtubules (MTs) either anti-α-Tubulin (Clone TAT-1; 1:2,000, CRUK) or anti-β-Tubulin (polyclonal; 1:200, CST), for detection of centrosomes either anti-γ-tubulin (polyclonal; 1:1000, Sigma) or anti-pericentrin (polyclonal; 1:500, Covance), and subsequently with highly cross-adsorbed Alexa Fluor 488, 568 or 647-conjugated anti-rabbit or anti-mouse secondary antibodies (1:1000, Molecular Probes), where appropriate. GFP-tagged proteins were detected in fixed samples solely using epi-fluorescence. All cells were mounted in Prolong Antifade with DAPI (Molecular Probes).

Statistical analysis

Statistical analysis was performed using Microsoft Excel and Origin 7.5. Unless indicated otherwise the p values shown were calculated using a two-tailed Student's t-test.

Fluorescence microscopy and analysis of fixed samples

Scoring of mitotic index, mitotic stage, chromosome congression errors and proportions of monopolar/bipolar spindles in fixed cells were performed using an Olympus BX51 microscope with UPLSAPO 40X2 0.95NA, UPLSAPO 60x 1.35NA and UPLSAPO 100x 1.4NA objective lenses. Illumination was achieved via a 300W Xenon light source for fluorescence with DAPI (49000), GFP (49002) and DsRed (49005) filters. Image capture was via a Photometrics Coolsnap HQ and Metamorph (Molecular Devices) software. For analysis of chromosome congression errors, cells with the majority of chromosomes clearly aligned at the metaphase plate and that had a robust mitotic spindle with 1 or more chromosomes which were clearly unaligned were counted as having chromosome congression errors, whereas those with all chromosomes aligned were counted as having normal chromosome congression. For Tiam1/Rac localisation and inter-centrosomal distance measurements, images were recorded with a Deltavision Core system (Applied Precision Instruments) based around an Olympus IX71 microscope with illumination achieved by white light LED and a 300W Xenon light source for fluorescence. The Sedat filter (Chroma, 89000) set was utilised for fluorescence imaging using Olympus PLAPO 100XO/TIRFM-SP 1.45 NA or UPLSAPO 60XO 1.35NA objective lenses, and image capture was via a Roper Cascade II 512B EMCCD camera and SoftWorx software (Applied Precision Instruments). Inter-centrosomal distance was measured in 3 dimensions using

SoftWorx, with a separation of less than 4 μm following NEBD considered as 'monopolar'. Images were processed with Imaris (Bitplane) and Photoshop 6.0 (Adobe) software.

Time-lapse microscopy

Time-lapse microscopy was performed using either the Deltavision system (described above) or an Incucyte system (Essen Instruments). Phase-contrast images were captured at 4 minute intervals using the Incucyte system (at 10 x magnification) under normal growth conditions (i.e. at 37^{o}C within a 5% CO_2 incubator). The mitotic duration (calculated as minutes from the first frame after NEBD to the first frame with evidence of cytokinesis and/or membrane blebbing) was analysed in Microsoft Office Picture Manager. For fluorescent time-lapse microscopy using the Deltavision system, cells expressing Histone-2B-GFP or β-Tubulin-GFP were plated in 35mm glass-bottom Petri dishes (MatTek Corporation), and during image capture cells were cultured in a permanently heated chamber with 5% CO_2. Images were recorded using an Olympus UPLSAPO 60XO 1.35NA lens with acquisition every 2 minutes over a depth of 10 μm (z-stacks at 1 μm intervals) for a total of 3-10 hours. Subsequent analysis of mitotic cells, including duration of early mitotic stages, was performed using Imaris software. For β-Tubulin-GFP expressing cells, subsequent calculation of centrosome separation rate was performed in Excel (Microsoft): for prophase (before NEBD), the period of separation was identified for each individual cell and the rate calculated from the slope of a line fitted to the relevant time-points. The pro-metaphase rate was calculated from the first 4 time-points after NEBD for all cells.

Supplemental References

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