

Supplementary Materials and Methods

Cell cultures, synchronisation, and reagents

Human cervical cancer HeLa cells and osteo-sarcoma U2OS cells were cultured in high-glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Immortalised human pigment epithelial cells hTERT-RPE-1 were cultured in DMEM/F12 (Invitrogen) supplemented with 10% FBS and 1% non-essential amino acid. All cells were cultured in a humidified 5% CO₂ incubator at 37°C. For the detection of protein level during the cell cycle, cells were synchronised using mimosine (M0253; Sigma), hydroxyurea (H8627, Sigma), Nocodazole (M1404; Sigma), or MG132 (47490; Merck).

Plasmids, transfection and RNA interference

Plasmids containing hMsd1 fragments were constructed using full-length hMsd1 cDNA (RG207526; from OriGene Technologies) as a template with appropriate PCR primer pairs. The amplified products were subcloned into pEGFP-C1 (Clontech). hMsd1-C-PACT was created by adding the PACT domain sequence (a gift from Fanni Gergely, Cancer Research UK Cambridge Institute and Sean Munro, University of Cambridge) at the C terminus of hMsd1-C using appropriate primer pairs. For transfection experiments, cells were first split to 70-90% confluency and then individual plasmid DNAs were transfected using Lipofectamine 2000 (Invitrogen). pMyc-dynamitin was a gift from Andrew Fry (University of Leicester).

Double-stranded siRNA oligonucleotides were synthesised with the sequences 5'-GACAGACAGUUACAAUGUA-3' (hMsd1 siRNA; Dharmacon), 5'-GGCUUUAACUAAUUAUGGA-3' (PCM1 siRNA; Dharmacon), or 5'-CGGUACAAUGAGUGUAGAA-3' (Ninein siRNA; Dharmacon). Control depletion was carried out using siGENOME non-targeting siRNA (Dharmacon). To deplete hMsd1, cells were also treated with two different siRNA oligonucleotides used by Barenz et al. (2013) (No.1=s42131: 5'-GCAUGUCUAAACUUACUAATT-3' and No.2=s42132: 5'-GGGACAAUCUUAUAGUGCATT-3') (Life Technologies Corp.)[§][1] (see Supplementary Fig S6). For RNAi experiments, cells were first transfected with 20-40 nM of dsRNA

using Lipofectamine RNAi-MAX (Invitrogen), followed by the second siRNA transfection 24 h later. Microscopic observation was performed 24 h after the second transfection (in total after 48 h) unless otherwise stated. For hMsd1 siRNA rescue experiments or expression of various hMsd1 constructs upon hMsd1 depletion, we constructed pEGFP-hMsd1-res that were resistant to hMsd1 siRNA, by introducing seven silent substitutions within the hMsd1 siRNA-target region. The hMsd1 siRNA target region, 5'- GAC AGA CAG TTA CAA TGT A-3', was changed to 5- GAT CGG CAA CTG CAG TGC A-3' using site-directed mutagenesis with the primers, 5'- gggaaatgattgggcttcaggaaagagataggcaactgcaatgtaagaacaggaattgcatcagc-3' and 5- gctgatgcaaattcctgttcttacattgcagttgcctatctcttctgaagcccaatcatttccc-3'. Cells were treated with hMsd1 siRNA for 48 h prior to transfection with hMsd1 constructs, and microscopic observation was performed 24 later. For time-lapse imaging, cells were mounted in L15 media supplemented with 10% FBS.

Immunofluorescence microscopy and image analysis

For immunofluorescence microscopy, cells were fixed with methanol at -20°C for 5 min and washed in PBS. After blocking in 3% BSA for 1 h at room temperature, cells were incubated with primary and secondary antibodies. These cells were then incubated at room temperature for 1 h and washed three times with 0.02% Tween 20 in PBS. DNA was visualised by the addition of DAPI (4,6-diamidino-2-phenylindole; Vector Lab.). During time-lapse imaging, cells were kept at $34-37^{\circ}\text{C}$ by a chamber heater. Images were taken using an Olympus IX71 wide-field inverted epifluorescence microscope with Olympus PlanApo 60 \times , NA 1.4, or UApo 40 \times , NA 1.35 oil immersion objectives. DeltaVision image acquisition software (softWoRx 3.3.0; Applied Precision Co.) equipped with Coolsnap-HQ digital CCD camera or Cascade EMCCD 512B camera (Roper Scientific) was used. The sections of images were compressed into a two-dimensional (2D) projection using the DeltaVision maximum intensity algorithm or sum intensity for microtubules. Deconvolution was applied before the 2D projection. Images were taken as 64 (interphase cells) or 80 sections (mitotic cells) along the z axis at 0.2-

µm intervals. Captured images were processed with Adobe Photoshop CS3 (version 10.0).

For immunofluorescence microscopy, the following antibodies were used; chicken anti-GFP (1:250, ab13970; Abcam), rabbit anti-SSX2IP (1:150, HPA027306; Sigma), mouse anti-SSX2IP (1:100, H00117178; Abnova), rabbit anti-PCM1 (1:300, sc67204; Santa Cruz Biotechnology), rabbit anti-γ-tubulin (1:250, T5192; Sigma), mouse anti-α-tubulin (1:250, T9026, Sigma), rabbit anti-Ninein (1:300, 602801; Biolegend), rabbit anti-Pericentrin (1:300, ab4448; Abcam), rabbit anti-EB1 (1:500, 610534; BD), mouse anti-acetylated tubulin (1:150, A21281; Sigma), mouse anti-Glu-tubulin (1:150, AB3201; Millipore), chicken anti-myc antibodies (1:300, A-10520; Molecular Probe). Secondary antibodies were Alexa Fluor 488-coupled anti-rabbit, Alexa Fluor 488-coupled anti-mouse, Alexa Fluor 488-coupled anti-chicken or Cy3-coupled anti-mouse antibodies (all used at 1:1,500).

For fluorescence signal intensity measurements, fluorescence signals were quantified using maximum intensity, after subtracting background signals in the vicinity of the fluorescent spot. ImageJ and softWoRx software were used for analysis.

Immunoprecipitation and immunoblotting

For coimmunoprecipitation, 5 mg cell lysate was incubated with 30 µl GFP-Trap agarose beads (ChromoTek) in lysis buffer (25 mM Tris-HCl, pH 7.0, 1 mM EDTA, 300 mM NaCl, 10% Glycerol, 1% NP-40, 1 mM DTT, 10 mM NaF, 25 mM DMSF and EDTA-free protease inhibitor tablet (Complete: Roche)) overnight at 4 °C. After washing with lysis buffer, the beads were denatured at 95 °C in NuPAGE buffer (NP0007; Invitrogen) and run on SDS-PAGE, followed by immunoblotting. For detection the following antibodies were used; mouse anti-GFP (1:2,000, 118144600001; Roche), rabbit anti-SSX2IP (1:1,000), rabbit anti-PCM1 (1:2,000), rabbit anti-γ-tubulin (1:1,000), rabbit anti-GCP2 (1:1,000, H00010844; Abnova), mouse anti-GCP3 (1:1,000, H00010426; Abnova), mouse anti-α-tubulin (1:5,000), rabbit anti-Ninein (1:1,000), rabbit anti-Plk1 antibodies (1:1,000, ab17057; Abcam).

Microtubule regrowth assay

siRNA-treated cells were incubated with 20 μ M Nocodazole for 2 h at 37 °C and then rinsed with medium, and prewarmed medium was added to induce MT regrowth. At appropriate time points (0, 5 and 30 min), cells were processed for immunofluorescence microscopy.

EB1 comet assay

HeLa cells were grown on coverslips and treated with Control or hMsd1 siRNA for 24 h. After transfection with pT7-EB1-Venus-N1 (a gift from Takahiro Matsusaka, Gurdon Institute, UK) for 16 h, time-lapse images were captured every 1 sec up to 1 min, and five frames were projected. Images were processed using Metamorph (Molecular Devices Corporation). To quantify the direction of EB1-Venus movements, EB1 comets were tracked during the time course while the comets moved straight^S[2]. The direction of an EB1 comet was defined by the direction of a vector from the position at which the tracking started to the point where it ended.

Spindle orientation assay

Procedures previously described were essentially followed^S[3,4] except that coverslips were not coated with fibronectin unless otherwise stated. Briefly, HeLa cells plated on coverslips were transfected with control or hMsd1 siRNA and arrested in metaphase with MG132 (5 μ M). Spindle orientation was analysed with the DeltaVision microscope system (softWoRx 3.3.0; Applied Precision Co.) by imaging z-sections (0.2 μ m per stack). Spindle angle was calculated with the sinus-function by measuring the distance in the x- and z-direction between the maximum intensities of the spindle poles using ImageJ and softWoRx software ($\theta = \sin^{-1}(Z/X)$). The same result, namely the emergence of tiled spindle upon hMsd1 depletion, was obtained in cells plated on fibronectin-coated coverslips^S[3] (Supplementary Fig S4D).

Quantification of aster, astral and spindle microtubule intensities in mitotic cells

For measurements of astral and spindle microtubule intensities, procedures previously described were essentially followed^S[4]. The image shown in Fig 4F is a cell treated with

control siRNA. The intensities of the spindle (I_{spindle}) and the total cell (I_{total}) were determined with ImageJ software. Relative astral MT intensity ($I_{\text{astral, rel}}$) was calculated by $[(I_{\text{total}} - I_{\text{spindle}}) / I_{\text{spindle}}]$ and the control value set to 1. The intensities of aster microtubules were measured, followed by subtracting background signal intensities and the control value set to 1 (Supplementary Fig. S4H).

Primary cilium induction

For primary cilium induction experiments, RPE-1 cells grown on coverslips were treated with control or hMsd1 siRNA. 24 h after transfection, cell were cultured under serum starvation conditions (0.3% FBS).

Zebrafish methodology

RNA encoding zebrafish Msd1-GFP (100 pg) and membrane-RFP (50 pg, red) was coinjected into the wild-type embryos and subjected to staining with γ -tubulin for the centrosome. For experiments using the Kupffer's vesicle, RNA encoding Msd1-GFP (100 pg) was injected into the wild-type embryo, and the embryo was fixed at the 8-somite stage for visualising the cilia. For morpholino (MO) experiments, translation-blocking morpholino was designed against zebrafish *msd1* (BC060934) and obtained from Gene Tools: 5'-CCAATCTCCCATGTCAACATTCAGC-3'. The MO (6.6 ng) was injected into the yolk of the 1-cell stage wild-type embryos, which were obtained from the fish facility at University College London. An antisense RNA DIG probe was synthesised using a DIG RNA-labelling kit (Roche) using cDNA for southpaw (*spaw*)^S[5]. Whole-mount in situ hybridization was performed as described previously^S[6]. Immunohistochemistry was carried out for early embryos (4 hpf) as described previously^S[7] and for cilia in the Kupffer's vesicles as described previously^S[8]. A monoclonal anti-acetylated γ -tubulin antibody (Sigma, T6793) was used for the staining of cilia.

Supplementary References

- S1. Barenz F, Inoue D, Yokoyama H, Tegha-Dunghu J, Freiss S, et al. (2013) The centriolar satellite protein SSX2IP promotes centrosome maturation. *J Cell Biol* **202**: 81-95.
- S2. Nagae S, Meng W, Takeichi M (2013) Non-centrosomal microtubules regulate F-actin organization through the suppression of GEF-H1 activity. *Genes Cells* **18**: 387-396.
- S3. Toyoshima F, Nishida E (2007) Integrin-mediated adhesion orients the spindle parallel to the substratum in an EB1- and myosin X-dependent manner. *EMBO J* **26**: 1487–1498.
- S4. Zhu M, Settele F, Kotak S, Sanchez-Pulido L, Ehret L, et al. (2013) MISPL is a novel Plk1 substrate required for proper spindle orientation and mitotic progression. *J Cell Biol* **200**: 773-787.
- S5. Long S, Ahmad N, Rebagliati M (2003) The zebrafish nodal-related gene *southpaw* is required for visceral and diencephalic left-right asymmetry. *Development* **130**: 2303-2316.
- S6. Barth KA, Wilson SW (1995) Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**: 1755-1768.
- S7. Shanmugalingam S, Houart C, Picker A, Reifers F, Macdonald R, et al. (2000) *Ace/Fgf8* is required for forebrain commissure formation and patterning of the telencephalon. *Development* **127**: 2549-2561.
- S8. May-Simera HL, Kai M, Hernandez V, Osborn DP, Tada M, et al. (2010) *Bbs8*, together with the planar cell polarity protein *Vangl2*, is required to establish left-right asymmetry in zebrafish. *Dev Biol* **345**: 215-225.