Supporting Information for

ASPM promotes ATR-CHK1 activation and stabilizes stalled replication forks in response to replication stress

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Other supporting materials for this manuscript include the following:

Datasets S1: mass spectrometry dataset.
**Supplementary Materials and Methods**

**Cell lines and culture conditions.**

Human HeLa, U2OS and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (1%) at 37°C in a 5% CO₂ humidified incubator. NPC cells were a kindly gift from Prof. Haiyun Gan at Shenzhen Institutes of Advanced Technology. NPC cells were grown in N2B27 with EGF/FGF (10 ng/ml each), on 0.2% gelatin-coated plates/flasks.

**Transient transfection**

Transient transfection to over-express plasmids in cell lines was carried out using polyethylenimine (PFI, Polyscience), according to the manufacturer’s instructions. siRNAs were transiently transfected using Lipofectamine® RNAiMAX transfection reagent (Invitrogen), according to the manufacturer’s instructions. All siRNA sequences are listed in SI Appendix, Table S1.

**ASPM KI and ASPM KO cell line generation**

Both KO and KI cell lines were generated using CRISPR-Cas9 genome-editing technology. For the ASPM KI, a single guide RNA (sgRNA) was designed in front of the start codon ATG within the ASPM first exon. The sgRNA (TCGAATCTGCCATGGCGAAC) -containing PX458 (Addgene, 48138) plasmid was co-transfected with pDsRed-FLAG-GFP donor plasmids (800 bp around the DSB site) into HeLa cells. After 48 h, both GFP- and RFP-positive single cells were selected by flow cytometry, diluted to single clones, and then confirmed by PCR and sequencing. For the ASPM KO, the sgRNA (GGCCCTAGACAACCCTAACG) -containing PX459 (Addgene, 48139) plasmid was transfected into HeLa cells. After 48 h, the cells were subcloned into 96-well plates after drug selection in the presence of 1 μg/mL puromycin for 24 h.

**Immunoblotting**

Cell lysates were prepared in 6×SDS loading buffer [62.5mM Tris-HCl, pH6.8, 2% sodium dodecyl sulfate, 0.05% bromophenol blue, 20% Glycerol, 5% β-mercaptoethanol] and boiled for 5-10min. Lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Roche) before blocking with skim milk and then blotting with the indicated antibodies.
Primary antibodies were incubated overnight at 4°C while secondary antibodies incubated for 1 h at room temperature. A Super Signal West Femto Substrate kit was used to visualize proteins after processing membranes using a FUJIFILM imaging system.

**Chromatin fractionation**

Cells were collected by trypsinization and suspended with Buffer A (10 mM HEPES pH 7.9, 0.34 M Sucrose, 20 % Glycerol, 1 mM dithiothreitol (DTT), 10 mM KCl, 1.5 mM MgCl₂) and 0.1 % Triton-X-100. The soluble cytoplasmic fraction (S2) was obtained after the first addition of Buffer A. The remaining nuclear fraction was washed with Buffer A (without 0.1 % Triton-X-100) before Buffer B (30 mM EDTA, 0.2 mM EGTA, 1 mM DTT) was added to the cells and kept on ice for 10 min. The soluble nuclear fraction was separated by centrifuge and the insoluble chromatin fraction was washed with Buffer B by centrifugation at 13000 rcf for 1 min at 4°C. Cell fractions were collected in the respective sample buffers before analysis by immunoblotting.

**Immunoprecipitation**

HEK293T cells were collected by trypsinization and washed with PBS (pre-cooled) twice. The cells were lysed in NETN buffer (0/150/400 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.5 % NP-40, 5 mM EDTA) with protease inhibitor for 30 mins. The supernatants were incubated with a primary antibody at 4°C overnight. Then, protein A/G Sepharose beads were used to capture the primary antibody-bound proteins for 1 h at 4°C. The beads were washed 3-5 times in NETN buffer before being transferred to the appropriate sample buffer, and analyzed by immunoblotting.

**Immunofluorescence**

U2OS or Hela cells were grown on top of coverslips and then fixed with 4% paraformaldehyde (PFA) for 10 min. The cells were washed with PBS for twice and then permeabilized with 0.5 % Triton-X-100 (in PBS) for 5 min. The cells were blocked with 2% BSA (in 0.1 % PBST) for 30-60 min before being incubated with primary and then secondary antibodies for 1 h each at room temperature (RT). The nuclei were stained with DAPI for 2 min and then images were captured under a DragonFly confocal imaging system (Andor).

**DNA fiber assay**
Cells were labeled with the 40 μM CldU for 30 min, followed by 100 μM IdU for another 30 min prior to incubation with/without the indicated drugs. Cells were harvested by trypsinization before 2 μL of the cell mixture (containing labeled and non-labeled cells) was loaded onto a slide and cell lysis solution (200 mM Tris-HCl, pH 7.5, 50 mM EDTA and 0.5% SDS). After air drying, the fibers were fixed in methanol:acetic acid (3:1) solution and denatured in 2.5 N HCl. Then, the slides were washed twice with 1×PBS and then blocked with 2% BSA (in PBS) before the addition of anti-BrdU primary and suitable secondary antibodies. Images were captured under a DragonFly confocal imaging system (Andor).

iPOND

Cells were labeled with 10 μM EdU for 15 min before treatment or not with the indicated drugs. The cells were then fixed with 1% PFA for 20 min at RT and then the crosslinks were quenched with 1.25 M glycine for 5 min using a table concentrator. Next, the cells were collected and permeated in permeabilization buffer for 30 min on ice. Before the next step, a click chemistry reaction was prepared to conjugate biotin to EDU. After rinsing with PBS, the nuclei were subjected to the click reaction with 10 μM biotin-azide for 1.5 h at 4°C under rotation, followed by washes with PBS and resuspension in cell lysis buffer (1% SDS in 50 mM Tris-HCl, PH 8.0). Then, the cells were sonicated for 10 min at high intensity (1s/5s on/off pulses) to obtain 100-300 bp fragments. Streptavidin-conjugated Dynabeads M-280 (Invitrogen) were added to the fragments and incubated at 4°C overnight. Finally, the samples were washed twice with lysis buffer and then washing buffer (containing 500 mM NaCl) before analysis by immunoblotting.

PLA assay

Cells were first labeled with 10 μM EdU for 15 mins at 37°C, with or without 1 h thymidine (10 μM) chase, and then subjected to 4 mM HU treatment for 3 h prior to two washes with PBS. The cells were fixed with 3.7 % formaldehyde for 10 min, then permeabilized with 0.2 % Triton X-100 for 5 min and blocked with 2 % BSA for 1h. Next, the cells were incubated with two primary antibodies at 4°C overnight. The subsequent procedures were carried out according to the manufacturer’s instructions of the Duolink In Situ Red Starter kit (Sigma-Aldrich). Images were captured using a DragonFly confocal imaging system (Andor).
Metaphase spreads

For metaphase enrichment, growing cultures were incubated with 0.4 μg/mL of the spindle poison Cochine (Sigma, catalog #C3915) at 37˚C for 4 h. The cells were then harvested using trypsin before 0.075M KCl was added and left to incubate at 37˚C for 15 min to allow the cells to swell. Then, 1 mL Carnoy’s Fixatives (methanol: glacial acetic acid 3:1) were slowly added while gently mixing by pipetting. A further Carnoy’s Fixatives was added and the cells were resuspended for fixation for 10 min at RT. These steps were repeated two additional times, and the cells were incubated overnight during the fixation. The supernatant was discarded each time. The fixed cells were gently resuspended and the cell densities were optimized onto a pre-cleaned slide. The slides were stained with Giemsa at 37˚C for 3 min, then rinsed with deionized water and air dried. The slides were scanned and images were captured under DragonFly confocal imaging system (Andor).

Colony formation assay

Cells were collected by trypsinization and counted such that 150-300 cells were added to each well of a 6-well plate. The cells were treated with the indicated drugs after 24 h. The cells were cultured for a further 12-15 days. Then, the cells were fixed with 4% PFA before 1× crystal violet staining at RT for 15 min.

BrdU labeling

BrdU incorporation was performed as previous described (1).

ssDNA/dsDNA pull-down assay

To generate ssDNA/dsDNA, biotinylated DNA oligomers (5′- AACCTGTCGTGCCAGCTGCA-biotin-3′) were first annealed to complementary ssDNA (5′- TGCAGCTGGCAGACAGTTAATGAATCGGCCAACGCGCGGGAGAGGCGGTTTGCGTATTGGGGCGCTTCTCCGCTCGAGCGGAGTC-3′) with molar ratio 1:4. The annealed ssDNA/dsDNA product (100 pmol) was first bound with streptavidin-M280 beads (Thermo Fisher), followed by extensive washing with binding buffer [10 mM tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 0.01% NP-40, and bovine serum albumin (BSA) 10 mg/ml] and then coated with bacterially-produced recombinant RPA32 and PRA70 (approximately 1 μg). After extensive washing with binding buffer, the beads carrying RPA-ssDNA/dsDNA were incubated in nuclear
extracts or bacterially-produced recombinant HIS-TopBP1, GST-RAD9 for 30 mins, followed by extensive washing with NETN buffer three times. The proteins bound to beads were then subjected to immunoblotting.

**Statistical analyses**

All statistical analyses were performed in Microsoft Excel and GraphPad Prism 8. If the data conformed to a normal distribution, unpaired t tests were used. One-way ANOVA was used when comparing more than two samples. Each experiment was repeated at least three times. p <0.05 was considered to indicate a statistically significant difference.
Fig. S1. ASPM is associated with DNA replication factors. (A) ASPM localizes at the mitotic spindle. Representative images of FLAG-GFP-ASPM knock-in (ASPM KI) HeLa cells generated by CRISPR-Cas9 technology. The localization of ASPM at the mitotic spindle localization was checked by co-labelling with α-tubulin. (B) The interaction between ASPM and the identified replication factors is DNA-independent. ASPM KI HeLa cell lysates were precipitated with FLAG antibody with/without benzonase, then analyzed by immunoblotting with indicated antibodies. * : non-specific signal. (C-E) ASPM is dispensable for S phase progression. siRNA-mediated ASPM knockdown
cells were incorporated with EdU (10 μM) for 1 h before FACS (D). The percentage of EdU-positive cells was quantified (E) and the ASPM knock-down effect was analyzed by immunoblotting with the indicated antibodies (C). Data represent the mean ± SEM from three independent experiments. n.s. p >0.05; one-way ANOVA. (F) and (G) ASPM deficiency has no impact on S phase progression. ASPM knockdown cells were incorporated with EdU (10 μM) for 1 h before immunofluorescence analyses (F). The percentage of EdU positive cells was then quantified (G). Data represent the mean ± SEM from three independent experiments. n.s. p >0.05; one-way ANOVA. (H) and (I) ASPM is dispensable for normal DNA replication progress. siRNA-mediated ASPM knockdown cells were tracked with CldU and IdU. The IdU/CldU tract length ratio for individual replication forks is shown (H). Data are representative of at least three independent experiments. n.s. p >0.05; one-way ANOVA.
Fig. S2. ASPM ensures CHK1 activation upon replication stress. (A) The cell cycle profile of the ASPM KO cells after 24 h HU (2mM) or APH (5 µM) treatment. (B) ASPM promotes ATR-CHK1 activation upon CPT treatment. Control and ASPM KO cells were incubated with different doses of CPT for 1 h before immunoblotting with the indicated antibodies. (C-E) ASPM facilitates ssDNA formation in response to replication stress. The effect of ASPM knock-down was analyzed by immunoblotting (C). Control or ASPM knockdown cells were incubated with BrdU for 24 h, then treated with HU for 1 h before immunofluorescence analyses under non-denaturing conditions with the indicated antibodies (D). The percentage of cells with BrdU foci (>10) in H2AX positive cells
was quantified (E). Data represent the mean ± SEM from three independent experiments. **p < 0.01; one-way ANOVA. (F) and (G) ASPM promotes RPA32 foci formation upon replication stress. Control or ASPM knockdown cells were treated with HU (2 mM) for 1 h, followed by immunofluorescence analyses with the indicated antibodies (F). The percentage of cells with RPA32 foci (>15) in γ-H2AX positive cells was quantified (G). Data represent the mean ± SEM from three independent experiments. n.s. p > 0.05, **p < 0.01; one-way ANOVA.
Fig. S3. ASPM facilitates RAD9/TopBP1 chromatin loading. (A) Associations among ASPM, RAD17, RAD9 and TopBP1 increased following HU treatment in a DNA-independent manner. ASPM KI HeLa cells were treated with HU for 1 h with/without benzonase before anti-FLAG immunoprecipitation (IP) and immunoblotting with the indicated antibodies. *: non-specific signal. (B) ASPM promotes RAD9 and TopBP1 chromatin loading upon replication stress. Control and siRNA-mediated ASPM knockdown cells were treated with HU (2 mM) for 1 h before chromatin fractionation to isolate the chromatin enriched fraction (P3) and immunoblotting with the indicated antibodies. (C) ASPM promotes RAD9 and TopBP1 chromatin loading upon replication stress. Control and ASPM KO cells were treated with HU (2 mM) for the indicated times before chromatin fractionation assay to isolate P3 and subsequent immunoblotting with the indicated antibodies. (D) ASPM facilities the RAD17–RAD9 association. HEK293T cells were first transfected with control or ASPM siRNAs for 24 h, then transfected with FLAG-VEC or FLAG-RAD17 for another 48 h. The cells were then treated with HU (2 mM) for 1 h, followed by immunoprecipitation (IP) with an anti-FLAG antibody and immunoblotting.
Fig. S4. ASPM protects nascent strand from degradation at stalled replication forks. (A) The effects of knocking down/knocking out ASPM was measured by immunoblotting with the indicated antibodies. Control, ASPM KO, and ASPM knockdown cells were labeled sequentially with CldU and IdU for 30 mins, then treated with HU (2 mM) for 5 h (left) or APH (5 µM) for 2 h, followed by conducting a DNA fiber assay (related to figure 5A). (B) Re-expression of FLAG-ASPM Δ18 in ASPM KO cells. Control, ASPM KO or re-expression of FLAG-ASPM Δ18 cells followed by immunoblotting with the indicated antibodies. (C) The effects of knocking down ASPM in HeLa, U2OS and HCT116 cells was measured by immunoblotting using indicated the antibodies (related to figure 5C). (D) and (E) ASPM is dispensable for normal DNA replication progress in neural progenitor cells (NPC) cells. shRNA-mediated ASPM knockdown NPC cells were sequentially tracked with CldU and IdU for 30 mins. The diagram delineates the experimental design and
representative images show the CldU and IdU replication tracks (top). The IdU track lengths are shown (bottom). The knockdown effect of ASPM was measured by immunoblotting using the indicated antibodies (D). (F) Measurement of nascent DNA degradation in ASPM NPC cells. shRNA-mediated ASPM knockdown NPC cells were sequentially labeled with CldU and IdU for 30 mins, then treated with HU (2 mM) for 5 h before DNA fiber assay. The diagram delineates the experimental design and representative images show the CldU and IdU replication tracks (top). The extent of nascent DNA degradation using the ratios of IdU track compared to CldU track was plotted (bottom). Data are representative of at least three independent experiments. n.s. p >0.05; one-way ANOVA. (G) The effects of knocking down SMARCAL1, ZRANB3 and HLTF in HeLa cells were measured by immunoblotting with the indicated antibodies (related to figure 5F). Data are representative of at least three independent experiments. **p <0.01; one-way ANOVA.
Fig. S5. High expression of ASPM is associated with unfavorable prognosis in multiple types of cancer. (A-E) The survival plots associated with high ASPM high expression in liver hepatocellular carcinoma (LIHC). n(high)=181, n(low)=181 (A); lung adenocarcinoma (LUAD). n(high)=239, n(low)=239 (B); brain lower grade glioma (LGG). n(high)=256, n(low)=256 (C); adrenocortical carcinoma (ACC). n(high)=38, n(low)=38 (D); and pancreatic adenocarcinoma (PAAD). n(high)=88, n(low)=87 (E).
| Name          | Oligonucleotide’s sequence       | Region       |
|--------------|---------------------------------|--------------|
| siCTR        | CGUACGCGGAUACUUCGA               | Nonspecific  |
| siASPM 1#    | GUGGUGAAGGUGACCUUC              | CDS          |
| siASPM 2#    | AUGCUAACAGCAGUUAU                | CDS          |
| siATR        | CCUGUGAUGUUGCUUGA               | CDS          |
| siATRIP      | AAGGUCACAGAUAAUAGAU             | CDS          |
| siRAD9       | GUCUUUCUGUCUGCUUC               | CDS          |
| siRAD17      | CAGACUGGGUUGACCCCAU             | CDS          |
| siTopBP1     | AGACCUAUGUAUCCAGA               | CDS          |
| siDNA2       | GUAACUUGUUAAUUGACA              | CDS          |
| siMRE11      | CGACUGCGAGUGGACUAU              | CDS          |
| siCtIP 3#    | CGAAUCUAGAUGCACAAA              | CDS          |
| siEXO1       | GCCUGAGAAUAAUUGUCU              | CDS          |
| siSMARCAL1   | GCUUUGACCUCUUAGCAA              | CDS          |
| siZRANB3     | CAAGAGAUAUCAUCGAAU              | CDS          |
| siHLTF       | GGUCUUGGGCUUAGCUA               | CDS          |
| shASPM 1#    | GCGGTCACTACAGTCTCTAAAA          | CDS          |
| shASPM 2#    | CCTGTGTTCCTCAGTCTCTT           | CDS          |
| sgASPM KI    | Forward: caccGTAGCTGCCATGGCCATGCAGATTCAc | (2)          |
|              | Reverse: aaacGTTCGCCATGGCCATGGCCATGCAGATTCAc |             |
| sgASPM KO    | Forward: caccGGCTAGACAACCTAACCCTAACG | (1)          |
|              | Reverse: aaacCGTTAGGTTGTCTAGGGCC |             |
| Primary Antibodies | Species | Source    | Identifier     |
|--------------------|---------|-----------|----------------|
| Anti-ASPM          | Rabbit  | Bethyl    | A300-BL1615    |
| Anti-β-actin       | Rabbit  | Sigma-Aldrich | Cat# A5441    |
| Anti-HA            | Rabbit  | Bethyl    | Cat# A190-208A |
| Anti-Histone H3    | Rabbit  | Abcam     | Cat# AB1791    |
| Anti-His-tag       | Mouse   | MBL       | Cat# D2913     |
| Anti-GST-tag       | Mouse   | MBL       | Cat# M2093     |
| Anti-FLAG-M2       | Mouse   | Sigma-Aldrich | Cat# F1804    |
| Anti-RPA32         | Rabbit  | Bethyl    | Cat# A300-244A |
| Anti-p-RPA32(S33)  | Rabbit  | Bethyl    | Cat# A300-246A |
| Anti-IgG Rabbit    | Rabbit  | ABclonal Science | Cat# AC005 |
| Anti-IgG Mouse     | Mouse   | ABclonal Science | Cat# AC011 |
| Anti-p-CHK1(Ser345)| Rabbit  | Cell Cignaling Technology | Cat# 2348 |
| Anti-CHK1          | Rabbit  | Santa Cruz Biotechnology | Cat# B1516 |
| Anti-TopBP1        | Rabbit  | Bethyl    | Cat# A300-111A |
| Anti-RAD9          | Rabbit  | Abcam     | Cat# AB70810   |
| Anti-RAD17         | Rabbit  | AbCam     | Cat# A5359     |
| Anti-BrdU          | Rat     | Abcam     | Cat# AB6326    |
| Anti-BrdU          | Mouse   | BD Biosciences | Cat# 347580   |
| Anti-ATR           | Rabbit  | Bethyl    | Cat# A300-138A |
| Anti-ATRIP         | Rabbit  | Cell Signaling Technology | Cat# 2737 |
| Anti-RPA70         | Rabbit  | Bethyl    | Cat# A300-241A |
| Anti-PCNA          | Rabbit  | Abcam     | Cat# AB92552   |
| Anti-BRCA1         | Rabbit  | Santa Cruz Biotechnology | Cat# sc-6954 |
| Anti-SMARCAL1      | Rabbit  | Abcam     | Cat# sc-376377 |
| Anti-ZRANB3        | Rabbit  | Bethyl    | Cat# A303-033A |
| Antibody Name                | Species | Source      | Cat#               |
|-----------------------------|---------|-------------|-------------------|
| Anti-HLTF Rabbit Abcam      | Rabbit  | Abcam       | Cat# AB17984      |
| Anti-DNA2 Rabbit Abcam      | Rabbit  | Abcam       | Cat# AB96488      |
| Anti-EXO1 Rabbit ABclonal   | Rabbit  | ABclonal    | Cat# A6810        |
| Anti-CtIP Rabbit Bethyl     | Rabbit  | Bethyl      | Cat# A300-488A    |
| Anti-MRE11 Rabbit Bethyl    | Rabbit  | Bethyl      | Cat# A300-181A    |
| Anti-RFC1 Rabbit Bethyl     | Rabbit  | Bethyl      | Cat# BL274        |
| Anti-RFC2 Goat Bethyl       | Goat    | Bethyl      | Cat# BL274G       |
| Anti-RFC4 Goat Bethyl       | Goat    | Bethyl      | Cat# A300-145A    |
| Anti-p-T1989 ATR Rabbit Abcam| Rabbit  | Abcam       | Cat# ab223258     |
| Anti-ATR Rabbit Bethyl      | Rabbit  | Bethyl      | Cat# A300-138A    |

| Secondary antibodies        | Species | Source      | Identifier               |
|-----------------------------|---------|-------------|--------------------------|
| Peroxidase AffiniPure Rabbit| Rabbit  | Jackson     | Cat# 711-035-152; RRID: AB_10015282 |
| Donkey anti-rabbit IgG (H+L)| Rabbit  | ImmunoResearch | RRID: AB_10015282         |
| Peroxidase AffiniPure Mouse | Mouse   | Jackson     | Cat# 115-035-166; RRID: AB_2338511 |
| anti-mouse IgG (H+L)         | Mouse   | ImmunoResearch | RRID: AB_2338511         |
| Fluorescein (FITC)-AffiniPure Mouse | Mouse | Jackson     | Cat# 115-095-003; RRID: AB_2338589 |
| Goat Anti-Mouse IgG (H+L)   | Mouse   | ImmunoResearch | RRID: AB_2338589         |
| Alexa Fluor® 594 D∞-G(H+L)  | Rabbit  | Jackson     | Cat# 711-585-152; RRID: AB_2340621 |
| Donkey anti-Mouse Alexa Fluor 594 | Mouse | Life Technologies | A21203 |
| Donkey anti-Rat Alexa Fluor 488 | Rat    | Jackson     | 712-546-150             |
### Table S3. Drugs, reagents, and software used for this study

| Drugs        | Source                | Identifier                  |
|--------------|-----------------------|-----------------------------|
| Hydroxyurea  | Sigma-Aldrich         | CAS No. 127-07-1             |
| Aphidicolin  | Sigma-Aldrich         | CAS 38966-21-1              |
| Camptothecin | Selleck                | CAS No. 7689-03-4           |
| EdU          | Invitrogen             | Cat. no. E10187             |

#### Reagents

| Reagents                             | Source                | Identifier                  |
|--------------------------------------|-----------------------|-----------------------------|
| FLAG M2 Affinity Gel                 | Sigma-Aldrich         | Cat# A2220; RRID: AB_10063035|
| Protein A SEPHAROSE                  | General Electric      | Cat# 17061801               |
| Protein G SEPHAROSE                  | General Electric      | Cat# 17078001               |
| YF-594/647A Click-iT EdU Imaging Kits| US EVERBRIGHT         | Cat# C6017/C6018            |
| Lipofectamine-RNAiMAX Transfection Reagent | Thermo Scientific | Cat# 13778150               |

#### Software and algorithms

| Software and algorithms | Source                | Link                                      |
|-------------------------|-----------------------|-------------------------------------------|
| Image J                 | National Institutes of Health | https://imagej.nih.gov                     |
| FlowJo                  | Treestar              | https://www.flowjo.com/                   |
| GraphPad Prism 6.0      | GraphPad              | https://www.graphpad.com/                 |
| Fusion                  | Andor                 | N/A                                       |

### SI References

1. Xu S, et al. (2021) ASPM promotes homologous recombination-mediated DNA repair by safeguarding BRCA1 stability. *iScience* 24(6):102534.
2. Jiang K, et al. (2017) Microtubule minus-end regulation at spindle poles by an ASPM-katanin complex. *Nat Cell Biol* 19(5):480-492.