Human microcephaly ASPM protein is a spindle pole-focusing factor

Ami Ito, Elsa A. Tungadi, Tomomi Kiyomitsu, and Gohta Goshima†

Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan
†E-mail: goshima@bio.nagoya-u.ac.jp Phone: +81-52-788-6175

Nonsense mutations in the ASPM gene have been most frequently identified among familial microcephaly patients. Depletion of ASPM causes spindle pole unfocusing during mitosis in multiple cell types of Drosophila. However, it remains unknown whether human ASPM has a similar function. Here, using CRISPR-based gene knockout (KO) and RNA interference combined with chemical inhibitors and auxin-inducible degron, we show that ASPM functions in spindle pole organisation redundantly with the centrosomes and the kinesin-14 motor HSET in human tissue culture cells. Deletion of the ASPM gene alone did not affect spindle morphology or mitotic progression. However, when CDK5RAP2, the activator of centrosomal microtubule nucleation, was degraded in ASPM KO cells, the spindle poles were unfocused and mitosis was significantly delayed. HSET inhibition in ASPM KO cells also resulted in synthetic pole disorganisation. Similarly, a hypomorphic mutation identified in microcephaly patients caused spindle pole unfocusing in the absence of CDK5RAP2, suggesting this spindle pole defect as a possible cause of microcephaly.

Introduction

The most common cause of autosomal recessive primary microcephaly is a homozygous mutation of the ASPM gene (Abdel-Hamid et al., 2016; Bond et al., 2002; Bond et al., 2003; Tan et al., 2014). ASPM was originally identified in Drosophila as the orthologue Asp, whose mutation results in abnormal spindle formation (Ripoll et al., 1985). In the absence of Asp, the centrosomes are detached from the main body of the spindle, and spindle microtubules (MTs) are unfocused at the pole (Ito and Goshima, 2015; Morales-Mulia and Scholey, 2005; Saunders et al., 1997; Schoborg et al., 2015; Wakefield et al., 2001). Asp is concentrated at the spindle pole, the area enriched with the spindle MT minus ends. The current model is that Asp binds directly to the spindle MT ends using the middle region containing calponin homology domains and cross-links them each other, thus postulating Asp as a critical pole-focusing factor (Ito and Goshima, 2015). Recent studies have also shown that asp mutant flies have reduced brain size (Rujano et al., 2013; Schoborg et al., 2015), which was at least partly attributed to chromosome mis-segregation associated with unfocused spindle poles (Rujano et al., 2013).

The cellular function of human ASPM has been evaluated using RNA interference (RNAi)-mediated knockdown. Small interfering RNA (siRNA)-based knockdown in U2OS cells led to spindle misorientation, cytokinesis failure, reduction in mitotic cells, and apoptosis (Higgins et al., 2010), possibly through interacting with the citron kinase (Gai et al., 2016; Paramasivam et al., 2007). Another study observed downregulation of BRCA1 protein upon ASPM knockdown (Zhong et al., 2005). To the best of our knowledge, the pole-focusing defect commonly observed upon Asp depletion in Drosophila has not been reported in studies of human ASPM knockdown. However, RNAi has a general limitation in that the residual protein expression might suffice to fulfil the function of the target protein. Moreover, none of the previous RNAi studies of ASPM involved a rescue experiment in which full-length ASPM was ectopically expressed after endogenous ASPM depletion, leaving the possibility that some of the observed phenotypes were derived from off-target effects of the siRNAs utilised. The effect of mutations identified in microcephaly patients has also not been assessed using a cell culture model. Nevertheless, the lack of the spindle pole phenotype is surprising, given the result obtained in Drosophila.

In this study, we used CRISPR/Cas9-based knockout (KO) as well as RNAi to decipher the mitotic function of ASPM in the human HCT116 cell line. Our data provide the first demonstration that human ASPM is an important factor in spindle pole organisation, working redundantly with a centrosomal protein and kinesin-14. Furthermore, mutations identified in microcephaly patients similarly impair this function of ASPM.

Results

No mitotic abnormality upon ASPM KO in the human HCT116 cell line

To precisely assess the function of human ASPM, we aimed to delete the ASPM gene in HCT116 cells using CRISPR/Cas9-based genome editing. Since it was reported that all ASPM isoforms are translated from a common start codon in exon 1 (Kouprina et...
after centrosomal protein depletion/inhibition, indicating that centrosomes are not a prerequisite for pole focusing (Megraw et al., 2001; Wong et al., 2015). Nevertheless, centrosomes play a supportive role in spindle MT focusing perhaps by supplying additional MTs for the motors and MAPs for the interaction (Baumbach et al., 2015; Chavali et al., 2016; Goshima et al., 2005; Heald et al., 1997; Ito and Goshima, 2015; Mountain et al., 1999; Silk et al., 2009; Wakefield et al., 2001). We hypothesised that lack of the pole unfocusing phenotype in the absence of ASPM may also be partly due to the presence of a sufficient amount of centrosomal MTs in this cell line.

To test this possibility, we aimed to deplete another microcephaly protein, CDK5RAP2 (also known as CEP215), which attaches the γ-tubulin ring complex (γ-TuRC) to the centrosome and activates γ-TuRC-dependent MT nucleation, in the ASPM KO line; CDK5RAP2 depletion reduces centrosomal MTs (Bond et al., 2005; Choi et al., 2010; Fong et al., 2008). To this end, we utilised the auxin-inducible degron (AID) system (Nishimura et al., 2009). We tagged endogenous CDK5RAP2 with mCherry and mini-AID (mAID) sequences in the HCT116 cells possessing the gene encoding *Oryza sativa* (Os) TIR1, the auxin responsive F-box protein that constitutes a functional SCF ubiquitin ligase in human cells (Fig. S2A–C). In this cell line, OsTIR1 protein was induced by doxycycline hyclate (Dox) (Natsume et al., 2016), and then CDK5RAP2-mAID-mCherry was degraded rapidly upon auxin (indole-3-acetic acid; IAA) addition to the culture medium (Fig. S2D). We observed spindle dynamics after 24 h of auxin treatment in the presence or absence of ASPM (Fig. 3A, Movie 3). We also analysed two control samples for this experiment. In the external control, the cells were not treated with Dox or IAA (‘untreated’ in Fig. 3B).

In the internal control, CDK5RAP2 was not degraded in a subpopulation of the cells treated with Dox and IAA (‘CDK5RAP2 remains’ in Fig. 3B). In cells that had intact endogenous ASPM, bipolar spindles with focused poles were observed in 21 of 22 cases, and all went into anaphase with only a slight delay, even when CDK5RAP2-mAID-mCherry signals were hardly detected (Fig. 3A second row, 3B, NEBD to anaphase onset; 30 ± 8 min [±SD], n = 22). In sharp contrast, when CDK5RAP2-mAID-mCherry was depleted in the ASPM KO background, 66 of 83 cells exhibited unfocused spindle poles; spindle MTs were not persistently associated with the pole during metaphase (Fig. 3A bottom row, 3B). In these cells, anaphase onset was significantly delayed (NEBD to anaphase onset in 44 cells was >1 h). We concluded that ASPM is required for pole focusing.
when the centrosome function is compromised.

A recent report showed that CDK5RAP2 binds to HSET and promotes association of HSET with the centrosome (Chavali et al., 2016). Therefore, the effect of CDK5RAP2 depletion may be partly attributed to reduction of the HSET activity at the centrosome. However, we interpret that other defects such as reduction of centrosomal MTs (Choi et al., 2010; Fong et al., 2008) also contribute to the synthetic effect with ASPM KO, since, unlike HSET-inhibited cells, MT focusing was not stably maintained at the pole during metaphase in CDK5RAP2-depleted cells (Fig. 3A bottom row).

**RNAi depletion phenocopies CRISPR-based KO**

One possible reason for the lack of mitotic defects in the ASPM KO line is that suppressor mutations were introduced into another locus during the few weeks of KO line selection. To exclude this possibility, we depleted ASPM more rapidly from the cell using RNAi. We prepared four independent siRNA constructs, one of which was previously reported to give rise to abnormal spindle orientation and mitotic progression in U2OS cells after 72 h (Higgins et al., 2010) (Table S1). Immunostaining using anti-ASPM antibody indicated that all constructs efficiently knocked down ASPM protein after 48 h in HCT116 cells; the intensity of the residual ASPM signal was similar among the four constructs and to that of the KO line (Fig. 4A, B). We observed a strong toxic effect for one of the constructs (siRNA #2) and mitotic acceleration for another (siRNA #3), suggesting that these constructs had off-target effects on important proteins other than ASPM (Table S1). However, cell proliferation, spindle morphogenesis, and mitotic progression were not defective for the remaining two constructs, reminiscent of the results observed in the KO line. These results strongly suggest that rapid ASPM depletion also has little impact on spindle organisation.

To test whether the synthetic phenotype with CDK5RAP2 depletion is also observed for ASPM RNAi, we treated the cells with three ASPM siRNAs (excluding the toxic siRNA construct) and then degraded CDK5RAP2 with the AID system. Time-lapse imaging indicated that pole unfocusing was frequently caused by CDK5RAP2 degradation for each siRNA construct (Fig. 4C). These results support the conclusion that ASPM becomes critical only when the centrosome function is compromised.

**Microcephaly mutation in ASPM causes pole unfocusing in the absence of CDK5RAP2**

Homozygous mutations in the ASPM gene have been identified at various positions in microcephaly patients, almost all of which introduce a premature stop codon in the gene (Abdel-Hamid et al., 2016; Bond et al., 2002; Bond et al., 2003; Nicholas et al., 2009; Tan et al., 2014). The two most C-terminal mutations identified in three previous studies were located in front of the HEAT repeat motif: a nonsense mutation at amino acid (aa) 3,233 and a 1-bp deletion at aa 3,252, which introduces a stop codon at aa 3,261 (Fig. 5A). To test the effect of microcephaly mutations in spindle pole organisation, we inserted mClover next to the aa 3,232 residue to closely mimic a patient mutation (Fig. 5A, Fig. S3A, C); we selected this mutation, and not the most C-terminal mutation, as the former site was located next to the CRISPR-friendly sequences. As the control, mClover was inserted at the C-terminus of the ASPM gene (Fig. S3B). As expected, several replacement lines were obtained for both constructs. ASPM [1–3,232]-mClover showed indistinguishable localisation at the pole from that of the full-length ASPM-mClover, consistent with the case of *Drosophila* Asp where the HEAT repeat was shown to be unnecessary for MT minus-end localisation (Ito and Goshima, 2015). However, when CDK5RAP2 was depleted by the AID system, we observed a severe pole unfocusing phenotype for the ASPM [1–3,232]-mClover line, reminiscent of the results for the ASPM KO line (Fig. 5B, C; Movie 4). Thus, the mutation that mimics that of microcephaly patients impaired the function of ASPM in pole organisation. Since other mutations in patients were identified upstream of the introduced mutation, a similar pole-unfocusing phenotype is expected to be observed for all cases.

**Discussion**

The results described above established that human ASPM, like the *Drosophila* orthologue Asp, is a spindle pole-focusing factor. ASPM/Asp would localise to and cross-link adjacent MT minus ends. However, unlike Asp in *Drosophila*, the depletion of which gives rise to severe phenotypes in various cell types and causes brain size reduction as well as lethality (Ripoll et al., 1985; Rujano et al., 2013; Saunders et al., 1997; Schoborg et al., 2015; Wakefield et al., 2001), ASPM was not essential in the human HCT116 cell line (derived from a patient with colorectal carcinoma). Our data indicated that ASPM becomes indispensable for pole organisation when the activity of kinesin-14 or a centrosomal MT nucleation factor is suppressed; normally, kinesin-14 and CDK5RAP2 are active enough to mask the function of ASPM in this cell type. This scenario may hold true for many other cell types in mammals. In a mouse model, mutations in Asp cause a reduction in brain and testis/ovary sizes but do not cause lethality (Fujimori et al., 2014; Pulvers et al., 2010). In general, mutations in critical spindle assembly factors lead to severe developmental problem, which are often associated with early embryonic lethality, as mitosis is a crucial event throughout animal development (Aguirre-Portoles et al., 2012; Castillo and Justice, 2007; Silk et al.,...
2009; Watanabe et al., 2016). The lack of embryonic lethality caused by mutations in ASPM and other pole-organising factors linked to microcephaly (e.g. CDK5RAP2, SAS-4) may be attributed to the highly redundant nature of pole organisation in mammals. Supporting this idea, a cell type-specific polar defect has been observed for another pole-focusing factor, NuMA (Seldin et al., 2016; Silk et al., 2009). Thus, it is tempting to speculate that certain neuronal cell types in humans more heavily rely on ASPM for pole organisation than on other factors, and therefore a severe phenotype only appears in the brain of the patients.

A model for the cause of microcephaly postulates a defect in spindle orientation and asymmetric cell division (Feng and Walsh, 2004; Fish et al., 2008; Fish et al., 2006; Gai et al., 2016; Lizarraga et al., 2010; Thornton and Woods, 2009). Consistent with this idea, spindle orientation error was reported following ASPM RNAi in human U2OS cells (Higgins et al., 2010). However, depletion of other proteins required for spindle orientation (LGN, aPKCa) does not cause microcephaly in the mouse model (Imai et al., 2006; Konno et al., 2008; Megraw et al., 2011). Moreover, a recent study using a Sas-4 mutant mouse model suggested that microcephaly is caused by mitotic delay and apoptosis, independent of spindle orientation (Insolera et al., 2014). The results of the present study raise the possibility that spindle pole disorganisation is a cause of the severe mitotic delay that leads to microcephaly.

Materials and Methods

Plasmid construction Plasmid information is described in Table S4. pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene #44230 (Cong et al., 2013)) was used to construct CRISPR/Cas9 vectors according to the protocol of (Ran et al., 2013). PAM and 20-bp sgRNA sequences were selected by the Optimized CRISPR Design tool (http://crispr.mit.edu) (Table S2).

Cell culture and line selection The original HCT116 cell line and the HCT116 cells expressing OsTIR1 under the conditional Tet promoter and the puromycin-resistant gene (HCT116 Tet-OsTIR1) were cultured in McCoy’s 5A medium (Gibco) supplemented with 10% foetal bovine serum (Gibco) and 1% penicillin-streptomycin-ampoterbin B suspension (Wako) (cells were obtained from Dr M. Kanemaki) (Natsume et al., 2016). Cells were maintained in a 37°C humid incubator with 5% CO₂. Effectine (Qiagen) was used for plasmid transfection. For the construction of the ASPM KO line, a CRISPR/Cas9 plasmid (pA112) with sgRNA and SpCas9 sequences, and a donor plasmid (pA114) harbouring the neomycin-resistant gene flanked by homologous sequences were transfected into the original HCT116 cell line. The complete ASPM KO line (KO⁺) was selected by co-transfection of the following two plasmids into the original HCT116 cell: a CRISPR/Cas9 plasmid (pA119) that contains two sets of sgRNA and SpCas9 targeting the N- and C-terminal regions, and a donor plasmid (pA118) that has the neomycin-resistant gene flanked by homologous sequences. mAID-tagged cell lines expressing CDK5RAP2-mAID-mCherry were generated according to the procedures described in (Natsume et al., 2016). In brief, the HCT116 Tet-OsTIR1 line was transfected with a CRISPR/Cas9 plasmid (pTK478) and a donor plasmid (pTK472) carrying mAID-mCherry and a hygromycin-resistant gene flanked by homologous sequences. The ASPM-KO/CDK5RAP2-mAID-mCherry line was selected by co-transfection of the CRISPR/Cas9 plasmid (pA112) and the donor plasmid (pA114) into the CDK5RAP2-mAID-mCherry expression line. ASPM-mClover and ASPM [1-3232]-mClover lines were selected by co-transfection of the CRISPR/Cas9 plasmid (pA113, pA121) and the donor plasmid (pA110, pA120) harbouring mClover and neomycin-resistant genes flanked by homologous sequences into the CDK5RAP2-mAID-mCherry expression line. Two to three days after transfection, the cells were plated on 10-cm culture dishes and cultured in selection medium containing 200 µg/ml hygromycin (Wako). 1 µg/ml puromycin (Wako), and/or 800 µg/ml G-418 (Roche) for 10–18 days. Single colonies were picked up for further selection in a 24-well plate. To confirm homozygous insertion, genomic DNAs were prepared from drug-resistant clones for most lines, whereas the cells were directly used as PCR templates for checking the ASPM-mClover lines (full-length and C-terminal truncation). PCR was performed using Tks Gflex DNA polymerase (TaKaRa) and corresponding primers (Table S3), as described by (Natsume et al., 2016). Proper tagging of mClover to ASPM was also verified by DNA sequencing after PCR.

mAID To degrade mAID-tagged CDK5RAP2, the cells were treated with 2 µg/ml Dox (Sigma) for 24 h to induce the expression of OsTIR1, and with 500 µM IAA (Wako) for 24 h to induce the degradation of CDK5RAP2-mAID-mCherry (Natsume et al., 2016).

RNAi siRNA transfection was carried out using Lipofectamine RNAimax (Invitrogen). In the ASPM depletion experiment, HCT116 cells were incubated with 13.3 nM ASPM or control luciferase siRNA for 42 h (live imaging) or 48 h (immunostaining). The primers for siRNA constructs are shown in Table S1 (note that we observed a possible off-target effect for at least two of the constructs). For live imaging after mAID-tagged CDK5RAP2 degradation, Dox and IAA were added to the culture at 24 h after siRNA transfection.

Microscopy, image analysis, and data presentation Live imaging was performed using spinning-disc confocal microscopy with a 60× 1.40 NA objective lens. A CSU-X1 confocal unit (Yokogawa, Japan) attached to a Nikon Ti inverted microscope, EMCCD camera ImagEM (Hamamatsu, Japan) that was cooled (~80°C) with a chiller (Julabo USA Inc.), and a stage chamber were used for image acquisition (37°C, 5% CO₂). Characterisation of the phenotypes of fixed samples was performed with the Nikon Ti inverted microscope attached to the EMCCD camera Evolve (Roper). Three objective lenses (100× 1.49 NA, 100× lens 1.45 NA, or 40× 1.30 NA) were used. The microscopes and attached devices were controlled using Micromanager. Images were analysed with ImageJ.
For live imaging, the MTs were stained with SiR-Tubulin (30 nM; Spirochrome) for >1 h prior to image acquisition. For HSET inhibition, MG132 (10 µM; Wako) was first added to the culture medium together with SiR-Tubulin at >1 h prior to imaging, and then CW069 (200 µM; Selleck Chemicals) was supplied. Immunostaining was performed according to the standard procedure using 6.4% paraformaldehyde, and anti-tubulin (YOL1/34, 1:500, rat monoclonal, AbD Serotec) and anti-ASPM (1:1,000, rabbit polyclonal, antigen [aa 3,425–3,477], Bethyl Laboratories, Inc.) antibodies. DNA was stained with DAPI. The fluorescence intensity of polar ASPM was measured from a single-plane image of the immunostained samples, with measurements taken from the cell’s most in-focus pole. Background intensity was subtracted from each measurement. Multiple images presented in a comparative manner in figures and movies were acquired and processed in an identical manner, except for those in Fig. 4A, in which SiR-tubulin staining in the ASPM #2 sample was stronger than the others, perhaps due to massive cell death induced by this specific siRNA; thus, the images were acquired with a shorter laser exposure for this particular sample. Graphical presentation of the data and statistical analysis were performed with the GraphPad Prism 6.0 software.

Immunoblotting Protein extracts were prepared according to a previously described protocol (Ito and Goshima, 2015). In brief, the cells were treated with the Cytobuster (EMD Millipore) solution, which also contained benzonase, dithiothreitol, and protease inhibitors (30 min on ice), followed by addition of the urea-containing solution (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 4 M urea, and 2-mercaptoethanol) at room temperature (5 h). Immunoblotting was carried out with anti-CDK5RAP2 (1:10,000, rabbit polyclonal, Abcam).

Acknowledgements

We are grateful to Toyoaki Natsume and Masato Kanemaki (National Institute of Genetics, Japan) for reagents and technical advice, and to Momoko Nishina (Nagoya University, Japan) for technical assistance. This work was supported by the Uehara Memorial Foundation and Takeda Science Foundation (to G.G.). A.I. is a recipient of a JSPS pre-doctoral fellowship. The authors declare no competing financial interests.

Supplementary Movie legends

Movie 1. Normal spindle formation and mitotic progression after ASPM KO
SiR-tubulin images were acquired every 3 min with spinning-disc confocal microscopy. Images were acquired with 5 z-sections (separated by 3 µm) and displayed after maximum projection. Time 0 corresponds to the timing of nuclear envelope breakdown. The images of two ‘incomplete’ KO lines (#4 and #23) and two ‘complete’ KO lines (#25 and #27) are presented.

Movie 2. HSET inhibition induced spindle pole splitting in the absence of ASPM
SiR-tubulin images were acquired every 3 min with spinning-disc confocal microscopy. Images were acquired with 5 z-sections (separated by 3 µm) and displayed after maximum projection. Time 0 corresponds to the timing of CW069 (HSET inhibitor) addition. Two ASPM KO lines (#4 and #23) were used.

Movie 3. CDK5RAP2 degradation impairs spindle pole focusing and mitotic progression in the absence of ASPM
Images were acquired every 3 min with spinning-disc confocal microscopy. Green, CDK5RAP2-mAID-mCherry; magenta, Sir-Tubulin. Images were acquired with 5 z-sections (separated by 3 µm) and displayed after maximum projection. Time 0 corresponds to the timing of nuclear envelope breakdown.

Movie 4. Microcephaly mutation of ASPM impairs spindle focusing and mitotic progression in the absence of CDK5RAP2
Images were acquired every 5 min with spinning-disc confocal microscopy. Yellow, CDK5RAP2-mAID-mCherry; magenta, Sir-Tubulin; cyan, ASPM-mClover (full-length or C-terminal truncation that mimics microcephaly mutations). Images were acquired with 5 z-sections (separated by 3 µm) and displayed after maximum projection. Time 0 corresponds to the timing of nuclear envelope breakdown.

References


Table S1. Primers for RNAi

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequences</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM -1</td>
<td>UGCCAUGGUGCAACUUGCU</td>
<td></td>
</tr>
<tr>
<td>ASPM -2</td>
<td>GCACAUAUAGAGGUAAACUGGACUGUGC</td>
<td>HSS138109 (Invitrogen)</td>
</tr>
<tr>
<td>ASPM -3</td>
<td>GCGCUUUAGGCGUAAACAGACGAGGC</td>
<td>HSS138110 (Invitrogen)</td>
</tr>
<tr>
<td>ASPM -4</td>
<td>GGAGAAAUCUCUCGACGUCUUCUCUCUCC</td>
<td>HSS138111 (Invitrogen)</td>
</tr>
<tr>
<td>ASPM -1</td>
<td>UGCCAUGGUGCAACUUGCU</td>
<td></td>
</tr>
<tr>
<td>ASPM -2</td>
<td>GCACAUAUAGAGGUAAACUGGACUGUGC</td>
<td>HSS138109 (Invitrogen)</td>
</tr>
<tr>
<td>ASPM -3</td>
<td>GCGCUUUAGGCGUAAACAGACGAGGC</td>
<td>HSS138110 (Invitrogen)</td>
</tr>
<tr>
<td>ASPM -4</td>
<td>GGAGAAAUCUCUCGACGUCUUCUCUCUCC</td>
<td>HSS138111 (Invitrogen)</td>
</tr>
</tbody>
</table>

Table S2. sgRNA sequences for CRISPR/Cas9-mediated genome editing

<table>
<thead>
<tr>
<th>Gene</th>
<th>sgRNA</th>
<th>PAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM (N-terminus)</td>
<td>TGCCATGGCGAAACGCGAG</td>
<td>TGG</td>
</tr>
<tr>
<td>ASPM (C-terminus)</td>
<td>CAAATGGTGATGGTACGCT</td>
<td>TGG</td>
</tr>
<tr>
<td>ASPM (mutation insertion)</td>
<td>CATTATGGAGAGGCTATTCT</td>
<td>TGG</td>
</tr>
<tr>
<td>CDK5RAP2 (C-terminus)</td>
<td>AAGAGACAGCGTGAGCTGCG</td>
<td>TGG</td>
</tr>
</tbody>
</table>

Table S3. PCR primers to confirm gene editing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Primer information</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM (N-terminus)</td>
<td>TTGCAGGGTATTGGGGCGTAG</td>
<td>Fig. 1A,S1B</td>
</tr>
<tr>
<td></td>
<td>AGGGTTGTCTAGGCGCGAG</td>
<td>Fig. 1A,S1B</td>
</tr>
<tr>
<td>ASPM (C-terminus)</td>
<td>CTCTCCACCTCTTTGACG</td>
<td>Fig. S1B,S3B</td>
</tr>
<tr>
<td></td>
<td>AATCATTTATGACATATTGTTATTACG</td>
<td>Fig. S1B,S3B</td>
</tr>
<tr>
<td>ASPM (mutation insertion)</td>
<td>TGGTCGATAATGCTGTG</td>
<td>Fig. S3A</td>
</tr>
<tr>
<td></td>
<td>AGAAATTGCTCCACCTGCG</td>
<td>Fig. S3A</td>
</tr>
<tr>
<td>CDK5RAP2 (C-terminus)</td>
<td>GGTAAATATTACGACAAAAAC</td>
<td>Fig. S2A</td>
</tr>
<tr>
<td></td>
<td>GGTATCGCCGGGATG</td>
<td>Fig. S2A</td>
</tr>
<tr>
<td>Neomycin</td>
<td>CCTAGGCTTTTGGAAAGATCGATC</td>
<td>Fig. S1B</td>
</tr>
</tbody>
</table>

Table S4. Plasmids constructed and used in this study

<table>
<thead>
<tr>
<th>lines</th>
<th>gRNA plasmid</th>
<th>Donor vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM-KO</td>
<td>pAI112</td>
<td>pAI114</td>
</tr>
<tr>
<td>ASPM-KO*</td>
<td>pAI119</td>
<td>pAI118</td>
</tr>
<tr>
<td>ASPM-mClover</td>
<td>pAI113</td>
<td>pAI110</td>
</tr>
<tr>
<td>ASPM[1-3232]-mClover</td>
<td>pAI121</td>
<td>pAI120</td>
</tr>
<tr>
<td>CDK5RAP2-mAiD-mCherry</td>
<td>pTK478</td>
<td>pTK472</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector information</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAI109</td>
<td>pAI109, which contained each ~800-bp DNA sequence of the ASPM gene flanking the stop codon in exon 28. The vector of this plasmid was pCR2.1-TOPO. The sequences corresponding to sgRNA and PAM were altered to encode amino</td>
<td>The BamHI fragment containing mClover and the neomycin-resistant gene were extracted from pMK277 (a gift from Dr. M. Kanemaki (Natsume et al., 2016)), and inserted into the BamHI site of pAI109.</td>
</tr>
</tbody>
</table>
was inserted in front of the stop codon.

The following two oligos containing 20-nt guide sequence were annealed and inserted into the BbsI site of pX330:
CACC-Gctgcatgcagaaccgcggcgag (top) and AAACCTgcgctgcgtgcgcatgcA (bottom)

pTK472

pUC57-amp, ~400-bp DNA sequences containing the last exon of the CDK5RAP2 gene (190,738–191,188: NC_000009) were synthesised. The STOP codon (tga) was mutated and replaced by (Gga GGA TCC) to insert a BamHI site. The sequences corresponding to sgRNA and PAM were deleted.

The BamHI fragment containing mAID-mCherry and the hygromycin-resistant gene was purified from pMK293 (a gift from Dr M. Kanemaki (Natsume et al., 2016)) and inserted into the BamHI site of the synthesised vector plasmid.

The following two oligos containing 20-nt guide sequence were annealed and inserted into the BbsI site of pX330:
CACC-Gagacacgcgtgagctcgcgg (top) and AAACCTgcgctgcgtgcgcatgcA (bottom)
Figure 1. The ASPM KO line shows no mitotic defects

(A, B) Construction of the ASPM KO line, in which the region surrounding the start codon at the first exon of the ASPM gene was replaced by the neomycin-resistant marker. Targeted integration of the marker cassette was confirmed by PCR (primers are indicated as magenta arrows). (C) Immunofluorescence microscopy confirmed the diminishment of the ASPM signal at the spindle pole in two selected KO lines (clones #4 and #23). (D) Spindle dynamics of ASPM KO lines. MTs were visualized by SiR-tubulin staining. Images were acquired with 5 z-sections (separated by 3 μm) and displayed after maximum projection. No abnormality was detected. See also Movie 1. (E) Mitotic duration with or without ASPM. Data represent the mean ± SEM. WT: 25 ± 1 (n = 33), KO #4: 22 ± 1 (n = 30, p > 0.05, Student’s t-test), KO #23: 27 ± 2 (n = 34, p > 0.3). Bars, 5 μm.
Figure 2. Spindle pole splitting upon double inhibition of ASPM and HSET

(A) Spindle morphology before and after addition of the HSET inhibitor (CW069, 200 μM). MTs were visualized by SiIR-tubulin staining. Arrowheads indicate split poles. Images were acquired with 5 z-sections (separated by 3 μm) and displayed after maximum projection. Two independent KO lines were analysed (clones #4 and #23). See also Movie 2. Bar, 5 μm. (B) Frequency of split poles after CW069 addition. The values of each experiment are indicated by coloured dots, whereas the mean values of 2–3 independent experiments are indicated by black bars. Overall, 7–24 spindles were analysed in each experiment.
Figure 3

(A) CDK5RAP2-mAID-mCh / MT

<table>
<thead>
<tr>
<th></th>
<th>NEBD</th>
<th>Prometaphase</th>
<th>Metaphase</th>
<th>Anaphase</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0 min</td>
<td>6</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>ASPM-KO #20</td>
<td>0 min</td>
<td>6</td>
<td>18</td>
<td>24</td>
</tr>
</tbody>
</table>

Residual CDK5RAP2

+          -
+          -

(B) +Dox, IAA (CDK5RAP2 remains)
+Dox, IAA (CDK5RAP2 disappears)
Untreated

Figure 3. Spindle pole unfocusing upon double depletion of ASPM and CDK5RAP2

(A, B) Spindle pole unfocusing in the absence of ASPM and CDK5RAP2. Treatment with Dox and IAA induced the degradation of CDK5RAP2-mAID-mCherry (marked ‘−’). Cells that were tolerant to this treatment are also displayed as the control (marked ‘+’). Two independent KO lines (#3 and #20) were analysed. Arrowhead and vertical lines indicate focused and unfocused poles, respectively; pole focusing was observed only transiently. Images were acquired with 5 z-sections (separated by 3 μm) and displayed after maximum projection. See also Movie 3. Experiments were performed twice, and the result of one experiment is displayed in the graph (n = 8–44). Bar, 5 μm.
Figure 4. RNAi-mediated knockdown phenocopies KO
(A, B) Efficient knockdown of ASPM using four independent RNAi constructs as indicated by immunofluorescence microscopy (A) and the signal intensity measurement (B). The data are shown as a box-and-whisker plot, with the median (line in the middle of box), upper and lower quartiles (the ends of box), maximum and minimum (whiskers), outlier (dots), and mean (marked ‘+’) values presented; n = 13–55. Experiments were performed twice, and the result from one experiment is displayed in the graph. (C) Spindle pole unfocusing by combined ASPM knockdown and CDK5RAP2 degradation. Two control images (± CDK5RAP2-mCherry signals) are also displayed. Images were acquired with 5 z-sections (separated by 3 μm) and displayed after maximum projection; n = 9–30. Experiments were performed twice, and the result from one experiment is displayed in the graph. Bars, 5 μm.
Figure 5

(A) mClover insertion after the aa 3,232 residue mimics the second-most C-terminally truncated protein identified among the microcephaly patients (Abdel-Hamid et al., 2016; Bond et al., 2003; Tan et al., 2014). As a control, mClover was tagged to the C-terminus of the full-length ASPM protein. (B, C) Spindle pole unfocusing caused by ASPM truncation in the absence of CDK5RAP2. Treatment with Dox and IAA induced the degradation of CDK5RAP2-mAID-mCherry (marked ‘–’). Cells that were tolerant to this treatment are also displayed as the control (marked ‘+’). Two independent truncation lines (clones #17 and #19) and control full-length ASPM-mClover lines (clones #8 and #22) were analysed. See also Movie 4. Images were acquired with 5 z-sections (separated by 3 µm) and displayed after maximum projection. N = 9, 47, 31 (full-length #8); 12, 19, 33 (full-length #22); 17, 38, 15 (mutant #17); and 6, 26, 23 (mutant #19). Bar, 5 µm.
Supplemental figure 1 (A-D)

A

ASPM

WT

ASPM-KO #4

B

ASPM locus (exon 1-28)

ASPM locus

Donor vector

Neomycin

Edited genome

C

<table>
<thead>
<tr>
<th>No template</th>
<th>WT</th>
<th>Heterozygous</th>
<th>WT</th>
<th>Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>a</td>
</tr>
<tr>
<td>c</td>
<td>d</td>
<td>c</td>
<td>d</td>
<td>c</td>
</tr>
</tbody>
</table>

KO<sup>C</sup> (2.1 kb in "a")

WT (0.4 kb in "b")

KO<sup>C</sup> (1.4 kb in "d")

WT (0.4 kb in "c")

D

DNA MT ASPM

WT

ASPM-KO<sup>C</sup>

#25

#27

ASPM

Low

High
**Figure S1. Construction of the complete ASPM KO line**

(A) In ASPM KO cells for which exon 1 was targeted, truncated ASPM protein was still weakly expressed and detected at the spindle pole. Note that signals in this figure were highly enhanced to best visualize the weak signals in the KO line (identical cells to those in Fig. 1C are displayed). (B, C) Construction of the complete ASPM KO line (KOC), in which the entire ASPM open reading frame was replaced by the neomycin-resistant marker. Targeted integration of the marker cassette was confirmed by PCR (primers are indicated as arrows in B). Expected bands are displayed on the left of (C). Asterisk in (C) indicates a non-specific band. (D) Complete ASPM KO (KOC) lines did not give rise to pole staining with anti-ASPM antibody. Images with highly enhanced signals are presented on the right. (E) Spindle dynamics of ASPM KOC lines. MTs were visualized by SiR-tubulin staining. Images were acquired with 5 z-sections (separated by 3 μm) and displayed after maximum projection. No abnormality was detected. Images of two clones (#25 and #27) are shown. (F) Mitotic duration with or without ASPM. Data are mean ± SEM. WT; 25 ± 1 (n = 71), KO #25; 24 ± 1 (n = 59, p > 0.4, Student’s t-test), KO #27; 25 ± 1 (n = 66, p > 0.8). Bars, 5 μm.
**Supplemental figure 2**

**A**

![Diagram of CDK5RAP2 tagging](image)

**B**

<table>
<thead>
<tr>
<th>Tet-OsTIR1</th>
<th>Parent</th>
<th>Homozygous #1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tagged (3.9 kb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>WT (0.4 kb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>CDK5RAP2-mAID-mCh #1</th>
<th>Parent</th>
<th>Homozygous #3</th>
<th>#20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KO (2.1 kb)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>WT (0.4 kb)</td>
<td></td>
</tr>
</tbody>
</table>

**D**

![Immunoblotting](image)

**Figure S2. Construction of the CDK5RAP2-mAID-mCherry (degron) line**

(A, B) Targeted integration of the marker cassette was confirmed by PCR (primers are indicated as magenta arrows). Clone #1 was used as the parent line of the following experiments. (C) ASPM was knocked out with the strategy described in Fig. 1A. (D) Confirmation of CDK5RAP2-mAID-mCherry protein degradation by Dox and IAA treatment. Immunoblotting using anti-CDK5RAP2 antibody and Coomassie staining of whole-cell extracts are presented. This blotting also confirmed the homozygous tagging of mAID-mCherry to CDK5RAP2.
Figure S3. Construction of full-length and truncated ASPM-mClover lines

(A) Construction of the truncated ASPM line, in which mClover and the neomycin-resistant gene were inserted at exon 24, which mimicked the second-most C-terminally truncated protein identified among microcephaly patients (aa 1–3,232). (B) As a control, mClover and the marker were integrated at the C-terminus of the full-length ASPM protein. (C) Targeted integration of mClover and the marker cassette was confirmed by PCR (primers are indicated as magenta arrows in A and B). The CDK5RAP2-mAID-mCherry line (clone #1) was used as the host of integration. Proper tagging of mClover (ASPM-mClover #8 and #22, ASPM [1-3,232 a.a.]-mClover #17 and #19) was also verified by DNA sequencing after PCR.