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Ensconsin/Map7 promotes microtubule growth and centrosome separation in Drosophila neural stem cells

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The mitotic spindle is crucial to achieve segregation of sister chromatids. To identify new mitotic spindle assembly regulators, we isolated 855 microtubule-associated proteins (MAPs) from Drosophila melanogaster mitotic or interphasic embryos. Using RNAi, we screened 96 poorly characterized genes in the Drosophila central nervous system to establish their possible role during spindle assembly. We found that Ensconsin/Map7 mutant neuroblasts display shorter metaphase spindles, a defect caused by a reduced microtubule polymerization rate and enhanced by centrosome ablation. In agreement with a direct effect in regulating spindle length, Ensconsin overexpression triggered an increase in spindle length in S2 cells, whereas purified Ensconsin stimulated microtubule polymerization in vitro. Interestingly, ens null mutant flies also display defective centrosome separation and positioning during interphase, a phenotype also detected in kinesin-1 mutants. Collectively, our results suggest that Ensconsin cooperates with its binding partner Kinesin-1 during interphase to trigger centrosome separation. In addition, Ensconsin promotes microtubule polymerization during mitosis to control spindle length independent of Kinesin-1.

Introduction

During mitosis, the mitotic spindle plays a crucial role in ensuring the correct segregation of chromosomes. One of the principal challenges faced by the cell during mitosis is the assembly of a powerful microtubule (Mt)-based machine mediating the efficient segregation of sister chromatids within a few minutes. Because of their role in defining the plane of cell division, mitotic Mts are also essential for brain homeostasis, a process linked to asymmetric stem cell division (Caussinus and Gonzalez, 2005; Wodarz and Näthke, 2007; Neumüller and Knoblich, 2009). The assembly of the spindle is complex and tightly regulated. The association of Mt-associated proteins (MAPs) with Mts and their subsequent dissociation play an essential role in mitotic spindle assembly. Mitotic spindle assembly and chromosome segregation thus require the complex spatial and temporal regulation of MAPs (Gadde and Heald, 2004).

Many MAPs have been identified in various model organisms. These proteins can be classified into two main categories: motor and nonmotor proteins. Through their direct effects on Mt dynamics, Mt sliding, and cross-linking, these MAPs are involved in several steps in mitosis: centrosome separation and positioning, the attachment of Mts to kinetochores (Kts), bipolar spindle formation, chromosome motion, and cytokinesis (Walczak and Heald, 2008). Mt polymerization is promoted by MAPs, such as TOGp/MAP215/msps (Cullen et al., 1999; Tournebize et al., 2000; Cassimeris and Morabit, 2004), and Mts are organized into a fusiform structure by motor proteins (Suwim et al., 1992; Wittmann et al., 1998; Goshima et al., 2005). Spindle size is controlled by the coordinated regulation of Mt end-binding proteins, such as CLASPs, depolymerizing...
kinesins, and the Mt minus end protein known as patronin (Gaetz and Kapoor, 2004; Maiato et al., 2004, 2005; Laycock et al., 2006; Goodwin and Vale, 2010).

This study focuses on Ensconsin/MAP7. We demonstrate, for the first time, the occurrence in Drosophila melanogaster neurons (NBs) of Ensconsin/MAP7-dependent interphase centrosome separation and spindle assembly pathways during mitosis.

Results and discussion

Ensconsin is a general MAP required for mitotic spindle assembly in the fly central nervous system (CNS)

We analyzed the Drosophila Mt interactome from 0–2-h-old embryos, which are characterized by rapid, synchronous cell divisions (mitotic embryos), with the goal of identifying new spindle assembly proteins. In parallel, we also investigated the Mt interactomes of older embryos (2–17 h old), in which most Ms have nonmitotic functions. Mt polymerization was induced by incubation with taxol, and the MAPs and Ms were harvested by centrifugation and resolved by SDS-PAGE (Fig. S1, A and B). We applied a high-throughput proteomics approach, leading to the high-confidence identification of 855 proteins in both samples (Fig. S1, C–E; and Table S2). Using spectral count, a semiquantitative measurement of protein abundance, we classified all the proteins identified as mitotic, general, or interphase MAPs on the basis of their enrichment profiles (Materials and methods).

We investigated the possible role in spindle assembly in vivo of 96 poorly characterized genes by applying RNAi to the fly CNS (Table S3 and Fig. S1 F; Dietzl et al., 2007; Mummery-Widmer et al., 2009; Neumüller et al., 2011). The new putative mitotic genes identified by this approach (Fig. S1 G and Table S1) included Ensconsin (CG14998). We used enscl mutants to investigate the role of this gene in mitotic spindle assembly (Fig. 1 B, bottom). We used three known Ensconsin mutant alleles: ensclΔC (C-terminal deletion), ensclΔN (N-terminal deletion), and ensclΔNull (full deletion, hereafter referred to as enscl; Sung et al., 2008). Western blot analyses showed all three mutant strains to have similar low levels of Ensconsin protein (see Fig. S3 A).

Enscl mutants had small brain lobes (Fig. 1 A) and a higher mitotic index (1.2 ± 0.5% in wild-type [WT] brains and 2.6 ± 1.0% in enscl mutant brains; n > 3,000 cells, 6 brains), which suggests that enscl mutation caused a mitotic delay due to prolonged spindle assembly checkpoint (SAC) activation. We tested this hypothesis with the double knockdown of Ensconsin and the SAC protein Mad2 by RNAi, with confirmation of the depletion of the protein (Fig. S2 A). Kt/Mt attachment defects were revealed by the large numbers of lagging chromatids during anaphase in double RNAi (6/30 anaphases) but not in cells subjected to mad2 or enscl RNAi (n > 40 anaphases; Fig. S2, B and C). In addition, flies producing double-stranded RNAs (dsRNAs) for either Ensconsin or Mad2 were viable, whereas flies producing both these RNAs died. This indicates that the SAC is required to prolong mitosis and to correct deleterious Kt–Mt attachment.

Ensconsin and Kinesin-1 mutant NBs display similar centrosome separation defects during interphase

A close examination of enscl mutant Nb spindles revealed that the centrosomes were not fully separated at prophase (Fig. 1 B, top; n = 20/91) and that metaphase spindles were shorter than WT spindles (Fig. 1 B, bottom; n > 300 spindles).

Time-lapse imaging of dividing Nb producing a GFP-tagged β-tubulin showed the time between nuclear envelope breakdown (NEBD) and anaphase onset to be 6.1 ± 0.9 min in WT cells and 8.0 ± 1.2 min in enscl mutants, which is consistent with SAC activation (Fig. 1, C and D, top; and Videos 1–3). We also confirmed that mean spindle length was significantly shorter in enscl mutants (8.2 ± 0.7 µm) than in the WT (10.7 ± 0.7; Fig. 1 D, bottom).

In Drosophila NBs, unlike most cell types, the centrosomes split and start to separate immediately after cytokinesis (Rebollo et al., 2007; Rusan and Peifer, 2007). The daughter centrosome is immobile, retains Mt nucleation activity, and is connected to the apical cortex, whereas the mother centrosome displays weak Mt nucleation and migrates to the opposite side of the cell. The mother centrosome organizes a second aster shortly before mitosis. The daughter centrosome is therefore inherited by the Nb and the mother centrosome is inherited by the ganglion mother cell (GMC; Conduit and Raft, 2010; Januschke et al., 2011). The angle between the Mt asters before NEBD reflects the efficacy of centrosome separation. It was found to be abnormally low in enscl mutants (Fig. 1 E, top; and Fig. 1 F; see also Video 2), for which 14% of Nbs presented mispositioned asters during prophase (Fig. 1 E, bottom; and Video 3). The mother centrosome is not visible (by GFP-tubulin staining methods) before NEBD. We therefore imaged Nbs expressing YFP-labeled centrosomes and GFP-labeled Ms between two consecutive mitoses (Rebollo et al., 2007; Januschke and Gonzalez, 2010). Centrosome splitting after cytokinesis was normal in enscl mutants, but the separation was incomplete, resulting in the apparent separation defect detected before NEBD (Fig. 2 A, interphase; and Videos 4 and 5). We observed no monopolar spindle formation, which suggests that the machinery required to separate centrosomes at NEBD is functional (Tanebaum and Medema, 2010). However, due to the late timing of separation, when the mother centrosome started to nucleate Ms at NEBD, it was often anchored to the apical cortex in place of the daughter centrosome and was therefore inherited by the Nb (8/25; Fig. 2 A and Video 5, green line).

Ensconsin is a coactivator of Kinesin-1 in interphase. We therefore investigated whether centrosome separation defects could be also detected after a loss of Kinesin-1 function (Sung et al., 2008; Metzger et al., 2012; Barlan et al., 2013). We found centrosome separation defects in khc27/khcΔ mutant brains, which lead to a small angle between centrosomes at NEBD but have no effect on spindle assembly (Fig. 2, B–E; and Videos 6 and 7). Centrosome inheritance was randomized and, in 50% of subsequent mitoses, the daughter centrosome ended up in the GMC (Fig. 2 D, bottom). These data demonstrate that early centrosome separation after cytokinesis is a prerequisite for correct centrosome segregation, a process in which Ensconsin and Kinesin-1
appear to be key players. Interestingly, spindle size and mitotic timing were normal in kinesin heavy chain (Khc)-depleted and mutant Nbs, which indicates a dependence of the short spindle phenotype on Ensconsin but not Kinesin-1 (Fig. 2 D).

Ensconsin is required for Mt growth in mitotic Drosophila Nbs

We analyzed the mitotic defects of the ensc mutants in detail by fully depolymerizing mitotic spindles from WT and ensc Nbs by cold treatment and monitoring Mt regrowth at 25°C (Fig. 3 A). In control tests, we observed large centrosomal asters 30 s after the return to 25°C conditions (Fig. 3 B, left, red arrows; n = 19).

We also detected asters around the main chromosome mass and discrete Kt fibers (Fig. 3 B, white arrows). At 90 s, the WT spindles had a normal, bipolar shape (n = 25). The ensc mutant Nbs displayed weak aster formation around the chromosomes and no obvious Kt fibers after 30 s (n > 30; Fig. 3 B, right). After 90 s, only short spindles with weak, disorganized Mt arrays were present (n = 24). Overall, our results suggest that Ensconsin contributes to the polymerization of spindle Mts. For confirmation of this role, we quantified Mt growth directly by tracking EB1-GFP comets at Mt plus ends in WT and ensc Nbs (Fig. 3 C and Video 8). Measurements of Mt dynamic parameters revealed that the mean rate of Mt polymerization was significantly lower...
Figure 2. **Centrosome separation is defective in kinesin-1 and ensC mutant Nbs.** (A) WT (top and Video 4) and ensC mutant (middle, bottom, and Video 5) Nbs expressing YFP-Asl (to label the centrosomes) and GFP-tubulin (to follow the daughter centrosome and Mts) were analyzed by time-lapse video microscopy. The green and blue lines follow the trajectories of the daughter and mother centrosomes, respectively. The two centrosomes did not separate fully in ensC mutants. For this reason, in several cases (8/25, 32%), when the mother centrosome acquired its Mt nucleation potential, it was attached to the apical cortex in place of the daughter centrosome, and was therefore inherited by the Nb rather than the GMC. (B) Prophase and metaphase in control or khc RNAi-treated Nbs (left) and Western blot analyses of Khc (top) and actin (bottom) after khc or control RNAi (right). (C) Prophase and metaphase in WT and khc27/khc63 mutant Nbs. In B and C, tubulin is shown in red, aPKC in blue, and phospho-histone H3 Ser10 in green. (D) Duration of mitosis and centrosome positioning in WT and khc27/khc63 mutant Nbs expressing Sas4-GFP (yellow) and Cherry-tubulin (gray). Mitosis lasted 7.2 ± 1.2 min in WT (n = 27) and 7.5 ± 1.5 min in khc27/khc63 (n = 48) cells. P = 0.652. Spindle length was 10.9 ± 0.8 µm in WT cells and 11.3 ± 1.8 µm in khc27/khc63 mutant cells. The daughter centrosome was inherited by the GMC in 24 of the 48 khc27/khc63 mutant cells (50%). Time is given in hours:minutes:seconds. In A and D, the green and the red arrows indicate the daughter and mother centrosomes, respectively. The circles indicate the contours of the cells. Bars, 10 µm. (E) Analysis of centrosome separation angle in WT (green) and khc27/khc63 (red) Nbs at NEBD.
(reduced by 12%) in the ensc mutant than in WT Nbs (Fig. 3 D), a difference sufficient to account for the short spindles observed in ensc mutants.

Spindle shortening and the duration of mitosis in the Nbs of ensc mutants are greater in the absence of centrosomes. Given the large number of short Mts nucleated by the centrosomes in the regrowth assay, we investigated whether the spindle formation defects observed in ensc mutants were greater in the absence of centrosomes. We therefore generated ensc and sas-4<sup>2214</sup> double mutant flies, the sas-4 gene being required for centrosome duplication (Basto et al., 2006), and monitored cell division (Fig. 4). In sas-4<sup>2214</sup> single mutants (Video 9, left), the spindle formed without centrosomes and mitosis lasted 9.0 ± 1.2 min (Fig. 4, A and C). In ensc,sas-4<sup>2214</sup> double mutants (Video 9, right), mitosis lasted significantly longer (10.1 ± 1.2 min) and the distribution of Mts was more strongly affected than in the single mutant (Fig. 4, A and C). The spindles were much shorter in the double mutant (6.7 ± 0.6 µm) than in the...
Figure 4. **Enscolin-dependent spindle assembly defects are enhanced by centrosome ablation.** (A) Cell division of WT, ensc, sas-4^{2214}, and ensc, sas-4^{2214} double mutant Nbs expressing β-tubulin–GFP. Time is given in minutes:seconds. The circles indicate the contours of the cells. Bar, 10 µm. (B) Analysis of mitotic spindle length in WT (green), ensc (red), sas-4^{2214} (blue), and ensc, sas-4^{2214} (purple) mutant Nbs. Spindle length was 10.7 ± 0.7 µm in the WT (n = 15), 8.2 ± 1.2 µm in the ensc mutant (n = 23), 8.5 ± 1.4 µm in the sas-4^{2214} mutant (n = 45), and 6.7 ± 0.7 µm in the ensc, sas-4^{2214} double mutant (n = 31). *, P = 2 × 10^{-6}; **, P < 5.2 × 10^{-8}. (C) Analysis of the duration of mitosis in WT (green), ensc (red), sas-4^{2214} (blue), and ensc, sas-4^{2214} (purple) mutant Nbs. The time between NEBD and anaphase onset was 6.1 ± 0.9 min for the controls (n = 23), 8.0 ± 1.2 min for the ensc mutant (n = 37), 9.0 ± 1.2 min for the sas-4^{2214} mutant (n = 48), and 10.0 ± 1.2 min for the ensc, sas-4^{2214} double mutant (n = 38). *, P = 1.8 × 10^{-3}; ***, P < 1 × 10^{-10}.
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and the mitotic spindle during Nb cell division and in early embryos (Fig. S3, C and D; and Video 10).

Consistent with the Ensconsin-dependent stimulation of Mt growth during M phase, S2 cells with higher Ensconsin levels formed longer spindles (10.9 ± 2.1 µm, n = 26) than control cells (7.8 ± 1.0 µm, n = 56; Fig. 5, A and B). In a complementary experiment, we assessed the impact of Ensconsin activity on Mt polymerization in vitro, using purified porcine brain tubulin and recombinant Ensconsin (Fig. 5 C) in a turbidity assay, to determine whether this protein could modify the Mt polymerization rate in vitro (Weis et al., 2010; Gaskin, 2011). Ensconsin stimulated Mt polymerization in a dose-dependent manner (Fig. 5 D). This function is entirely consistent with a lower Mt velocity in the deletion mutant and with a longer spindle length in conditions of Ensconsin overexpression.

In conclusion, our study reveals a dual role for Ensconsin.
First, during interphase, this protein promotes centrosome separation and positioning through cooperation with its binding partner, Kinesin-1. Second, it stimulates Mt growth during mitosis, independent of Kinesin-1.

Ensconsin is a mitotic spindle-associated protein that stimulates Mt growth in S2 cells and in vitro

We analyzed the distribution of Ensconsin by staining dividing Drosophila embryos with purified antibodies. Ensconsin colocalized with the mitotic apparatus during early development (Fig. S3 B). Furthermore, studies of a functional Drosophila line ubiquitously expressing the GFP-tagged Ensconsin confirmed the colocalization of Ensconsin-GFP with the Mt network and the mitotic spindle during Nb cell division and in early embryos (Fig. S3, C and D; and Video 10).

Consistent with the Ensconsin-dependent stimulation of Mt growth during M phase, S2 cells with higher Ensconsin levels formed longer spindles (10.9 ± 2.1 µm, n = 26) than control cells (7.8 ± 1.0 µm, n = 56; Fig. 5, A and B). In a complementary experiment, we assessed the impact of Ensconsin activity on Mt polymerization in vitro, using purified porcine brain tubulin and recombinant Ensconsin (Fig. 5 C) in a turbidity assay, to determine whether this protein could modify the Mt polymerization rate in vitro (Weis et al., 2010; Gaskin, 2011). Ensconsin stimulated Mt polymerization in a dose-dependent manner (Fig. 5 D). This function is entirely consistent with a lower Mt velocity in the deletion mutant and with a longer spindle length in conditions of Ensconsin overexpression.

In conclusion, our study reveals a dual role for Ensconsin. First, during interphase, this protein promotes centrosome separation and positioning through cooperation with its binding partner, Kinesin-1. Second, it stimulates Mt growth during mitosis, independent of Kinesin-1.

Figure 5. Ensconsin promotes Mt growth in cells and in vitro. (A) Ensconsin [Ensc] overproduction in mitotic S2 cells led to the formation of longer spindles. Ensconsin-GFP is green (gray in the panels on the right) and Mts are shown in red (gray in the middle panels). Exogenous Ensconsin-GFP is localized on the mitotic spindle during mitosis. Bar, 10 µm. (B) Analysis of mitotic spindle length in WT (n = 56) and Ensconsin-GFP-overexpressing mitotic S2 cells (n = 26). ***; P = 5.1 × 10⁻¹¹. (C) Recombinant Ensconsin purified, separated by SDS-PAGE, and stained with Coomassie blue. (D) Effect of Ensconsin on Mt polymerization in vitro. Purified porcine brain tubulin was mixed with polymerization buffer supplemented with either PBS or PBS containing Ensconsin on ice. Mt polymerization was induced by heating at 35°C. Absorbance at 350 nm was monitored to evaluate the formation of Mt polymers. After 28 min, the samples were shifted to 0°C to estimate protein aggregation. Ensconsin increased the rate of Mt polymerization and the total number of polymers in a dose-dependent manner (red curves), as shown by comparison with buffer alone (black curve).

sas-d²214 (8.5 ± 1.4 µm) and ensc single mutants (8.0 ± 0.6 µm; Fig. 4 B). In parallel, we repeated the Mt regrowth assay (Fig. 4 D). 1 min after the return to 25°C, long Mts were seen in the WT and the sas-d²214 single mutant, but not in the ensc single mutant and the ensc,sas-d²214 double mutant. Bipolar spindle assembly occurred within 3 min in flies lacking either sas-d²214 or ensc, but was severely compromised in the double mutant. These results suggest that the centrosome favors spindle assembly when Mt polymerization rates are low in mitotic ensc mutant cells.

(D) Mt regrowth assay in WT, ensc, sas-d²214, and ensc,sas-d²214 mutant Nbs. 1 and 3 min after the return to 25°C, numerous long Mts were detected in sas-d²214 Nbs. In the ensc,sas-d²214 double mutant, short Mts were found closely apposed to the mitotic chromatin mass. At 5 min, the spindles resembled Mt bundles rather than the bipolar structure seen in sas-d²214 mutants. aPKC is shown in blue, Mts (α-tub) are shown in red (and gray), and phospho-H3 is shown is green. Bar, 10 µm.
Human Ensconsin/MAP7/EMAP1 was first discovered two decades ago, and was described as a MAP that “ensconces” on Mts (Bulinski and Bossler, 1994). The data presented here reveal previously unidentified roles for this known MAP in the regulation of mitotic and interphase Mt-based processes. Further studies are required to determine how a single MAP can contribute to such different activities during the cell cycle.

Materials and methods

Mt co-sedimentation assay
Canton-S fly populations were amplified, maintained, and fed in population cages with fresh yeast on agar plates twice daily for 5 d. We collected O-2-8-h-synchronized embryos, a time period corresponding to syncytial division. In parallel, flies were allowed to lay eggs for 15 h and the collected embryos were left for an additional 2 h to allow the last embryos laid to reach the blastula stage. This protocol discriminates between the dividing/mitotic and differentiating/interphase stages. We disrupted 1 g of mitotic and interphase embryos by passage through a French press in a total volume of 2 ml of lysis buffer [LB: 20 mM HEPES, pH 7.4, 150 mM KCl, 1 mM MgCl₂, 1 mM EDTA, and 0.5% NP-40] supplemented with protease inhibitor and phosphatase inhibitor cocktails, prepared according to the manufacturer’s instructions (Roche). The crude extracts were filtered and centrifuged for 15 min at 15,000 × g. The resulting supernatant (1.2 ml) was centrifuged at 100,000 × g for 25 min at 4°C to remove insoluble materials. The mitotic and interphase supernatants were subjected to biased centrifugation, were supplemented with 1 mM GTP and 20 µM taxol, and incubated for 20 min at 25°C. The polymerized Mts and MAPs were then recovered by centrifugation for 20 min at 100,000 × g, and the supernatant (2–5 ml) was centrifuged and incubated for 20 min at 25°C. The polymerized Mts and MAPs were then recovered by centrifugation for 20 min at 100,000 × g and 25°C on a glycerol cushion (BRBB80: 80 mM Pipes, 1 mM EGTA supplemented with 40% glycerol, 1 mM GTP, and 20 µM taxol). The Mt pellets were washed gently twice with BRBB80, suspended in Laemmli sample buffer supplemented with 5% β-mercaptoethanol.

Liquid chromatography/tandem mass spectrometry (LC-MS/MS)
MAPs were resolved by SDSPAGE in 4–12% XT Bis-Tris polyacrylamide gradient gels (Bio-Rad Laboratories). The gel was stained with SYPRO Ruby gel stain according to the manufacturer’s instructions (Bio-Rad Laboratories). A GelImage charge-coupled device (CCD)-based imaging system (PerkinElmer) was used for image acquisition.

The lanes on the SDS-PAGE gel corresponding to mitotic and interphase/differentiating embryo extracts were cut into 20 gel slices per lane with a disposable lane picker (Gel Company). Gel slices were deposited in 96-well plates. A liquid handling device (MassPrep; Waters) was used, with sequencing-grade modified trypsin (Promega) according to the manufacturer’s instructions (Roche). The crude extracts were then dried by evaporation in a SpeedVac (Thermo Fisher Scientific).

Enrichment of mitotic and interphase embryos by passage through a French press in a total volume of 2 ml of lysis buffer [LB: 20 mM HEPES, pH 7.4, 150 mM KCl, 1 mM MgCl₂, 1 mM EDTA, and 0.5% NP-40] supplemented with protease inhibitor and phosphatase inhibitor cocktails, prepared according to the manufacturer’s instructions (Roche). The crude extracts were filtered and centrifuged for 15 min at 15,000 × g. The resulting supernatant (1.2 ml) was centrifuged at 100,000 × g for 25 min at 4°C to remove insoluble materials. The mitotic and interphase supernatants were subjected to biased centrifugation, were supplemented with 1 mM GTP and 20 µM taxol, and incubated for 20 min at 25°C. The polymerized Mts and MAPs were then recovered by centrifugation for 20 min at 100,000 × g and 25°C on a glycerol cushion (BRBB80: 80 mM Pipes, 1 mM EGTA supplemented with 40% glycerol, 1 mM GTP, and 20 µM taxol). The Mt pellets were washed gently twice with BRBB80, suspended in Laemmli sample buffer supplemented with 5% β-mercaptoethanol.

Fly strains
Flies were maintained under standard conditions at 25°C. Transgenic flies with the following genotypes were used for RNAi: TubGAL80*;69B-GAL4 and 69B-GAL4. Transgenic flies carrying UAS and hairpin sequences were obtained from the Bloomington Drosophila Stock Center (Dietzl et al., 2007). Flies expressing the full-length EB1-GFP and full-length Enscinsson-GFP constructs were used for time-lapse imaging. They were obtained from Best-Gene Inc. after Pelement-mediated transformation with the pUWG-EB1 and pUWG-Ensc vectors. The Enscinsson-GFP transgene conferred early rescue of the viability of ensc mutants. Asterless-YFP (Asl)-expressing flies (Reblolo et al., 2007) were provided by C. Gonzalez (Institute for Research in Biomedicine, Barcelona, Spain). Khc mutant flies carrying amino-acid substitutions have been described elsewhere (Djogaeva et al., 2012) and were provided by A. Guichet (Institut Jacques Monod, Paris, France), B. Saxton (University of California, Santa Cruz, Santa Cruz, CA), and A. Ephrussi (European Molecular Biology Laboratory, Heidelberg, Germany). A combination of KhcN3 (null allele) and KhcK27 (pyrophosphoric allele) was used in this study. The strains expressing mCherry-α-tubulin and GFP-Sas-4 were provided by R. Basto (Institut Curie, Paris, France) and M. Bettencourt-Dias (Instituto Gulbenkian de Ciencia, Oeiras, Portugal), respectively. The enscinsson mutant fly stocks enscC (C-terminal deletion), enscN (N-terminal deletion), and enscnull (full gene deletion, referred to as ensc) were described in the Results and discussion section, obtained by P-element excision and were provided by P. Rorth (Institute of Molecular and Cell Biology, Singapore; Sung et al., 2008). β-tubulin-GFP flies (Inoue et al., 2004) were provided by M. Savoini and D. Glover (University of Cambridge, Cambridge, England, UK). Jupiter-GFP and the sax-4Δ227 mutant (Basto et al., 2006) were obtained from the Bloomington Drosophila RNAi Center.
Stock Center. With the exception of Jupiter-GFP, which is a trap line with expression under the control of its own promoter (Morin et al., 2001), all GFP and CherryFP fusion proteins were ubiquitously produced under the control of the polyubiquitin promoter.

**RNAi in the brain**

**TUB-GAL80**; 69B-GAL4 females were crossed with transgenic males carrying UAS-RNAi constructs for the candidate genes. After 6 d at 20°C, the adults were removed and the vials were shifted to 20°C to induce RNAi for 3 d. Brains from five wandering third-instar larvae were then dissected and fixed for immunostaining. In parallel, at least five brains were squashed and stained with 1 µg/ml DAPI for the visualization of chromosome abnormalities (Donaldson et al., 2001).

**Production of recombinant proteins and antibody**

The vectors pET102-Ens-FL, pET102-Ens-EHR1, and pET102-Ens-EHR2 were transformed in *Escherichia coli* B121 (DE3). The production of the EnsFL, Ens-EHR1, and Ens-EHR2 fragments was induced by incubation with 1 mM IPTG for 4 h at 25°C. 6×-HIS–tagged proteins were purified on Ni-NTA resin (QIAGEN) and dialyzed overnight against PBS at 4°C. The purified Ensconsin EHR1 and EHR2 domains present in all Ensconsin isoforms were generally used for the immunization of rabbits for polyclonal antibody production (polyclonal antibody). For in vitro MT polymerization assays, Ens-fl was concentrated on Amicon concentrators (molecular weight cutoff 50 KD) to a final concentration of 30 µM in PBS (EMD Millipore).

**Immunofluorescence analysis**

S2 cells were plated on concanavalin A–coated coverslips and incubated for 1 h, then fixed by incubation in methanol for 10 min at −20°C. The fixed cells were processed for IF analyses as described previously (Romé et al., 2010). Slides were mounted in ProLong Gold antifading mounting medium (Life Technologies) and observed under an upright DMRXA fluorescence microscope (Leica) equipped with a 63× 1.3 NA objective lens. Images were acquired with a CCD camera (CoolSNAP HQ; Photometrics) and processed with Universal Imaging. For the analysis of spindle morphology in brain Nbs, we found that the following protocol fully preserved the Mt cytoskeleton during mitosis more effectively than the use of the standard PBS-derived fixative. For whole-mount CNS immunostaining, third-instar larval brains were dissected in testis buffer (TB: 183 mM KCl, 47 mM NaCl, 10 mM Tris, and 1 mM EDTA, pH 6.8; Bonaccorsi et al., 2011) and fixed by incubation in TB (TB supplemented with 10% formaldehyde and 0.01% Triton X-100) for 20 min at 25°C.

Fixed brains were rapidly washed once in PBS and twice in 0.1% Triton X-100 in PBS (PBST) for 10 min each time. The fixed tissue was incubated overnight at 4°C with primary antibodies in PBST supplemented with 1% BSA (PBSTB). The samples were washed twice, for 15 min each time, in PBST, and then incubated for 1 h at room temperature with secondary antibodies in PBSTB. They were washed twice, for 15 min each time, in PBST, then mounted on slides in Prolong Gold and observed with a SP5 confocal microscope (Leica) equipped with a 63× objective lens (NA 1.40). Images were acquired and processed with ImageJ software.

For the Mt regrowth assay in Nbs, we followed a method previously used to study *misato*, with several modifications (Mottier-Pavie et al., 2011). Brains were dissected in Schneider’s medium supplemented with 10% FCS and incubated on stainless steel slides with fat bodies dissected from *w* larvae (Siller et al., 2005). The preparations were sealed with mineral oil (Sigma-Aldrich). Images were acquired with a spinning-disk system mounted on an inverted microscope (Eclipse Ti; Nikon) equipped with a 60× 1.4 NA objective lens at 25°C. Z series were acquired every 30 s with 60 µs and a CCD camera (CoolSNAP HQ2; Photometrics) and a digital camera (iCMOS ORCA Flash 4.0; Hamamatsu Photonics) controlled by MetaMorph acquisition software version X. Images were processed with ImageJ software and are presented as maximum-intensity projections. The basis on their different Mt nuclear potentials after separation, after cytokinesis, or immediately before mitosis (Rebollo et al., 2007).

For Mt end tracking in Nbs, WT and ensce RNAi brains expressing EB1-GFP were gently squashed in 13.4 µl of fresh Schneider’s media (Invitrogen), between a slide and a 22 × 22-mm coverslip, and the excess medium was absorbed with filter paper. The preparation was sealed with mineral halocarbon oil 700 (Sigma-Aldrich) to immobilize it and to prevent cell movement during imaging. Mitotic Nbs were imaged every 0.5 s for 1 min at 25°C, with a 100× 1.4 NA objective lens.

At least 16 mitotic prometaphase Nbs were imaged for comet tracking. The Mt ends were tracked with the single-particle tracking routine available in Icy image analysis software (Olivo-Marin, 2002; Chenouard et al., 2013). The routine parameters were adjusted for the detection and tracking of most of the Mt ends. Nevertheless, because of the high density of Mts, we observed frequent erroneous merging of tracks corresponding to different Mt ends. These errors were filtered out with a customized Matlab (MathWorks) routine. The crude tracks were first broken at track steps characterized by an instantaneous speed of >0.78 µm/s, probably corresponding to erroneous jumps between different Mt tracks. They were also broken at positions at which a change in track direction of >60° was observed. We retained only the remaining filtered tracks corresponding to at least three time points and we estimated the growing speed for each track by averaging the instantaneous speeds along the track.

**Cell culture and transfection**

S2 cells were transiently transfected as described previously, and protein production was induced by incubation with 0.3 mM CuSO4 for 24 h (Romé et al., 2010).

**Mt self-assembly and turbidity assays**

A 40µM solution of tubulin purified from porcine brain was prepared in 1 mM GTP in BR88, and centrifuged for 10 min at 30,000 g, at 4°C, before assembly [Weis et al., 2010]. Tubulin polymerization was induced by incubating the samples at 35°C and was monitored turbidimetrically, at 350 nm, with a UVikon XS spectrophotometer (BioTek Instruments). We evaluated Ensconsin activity by adding various concentrations of the recombinant protein to tubulin (1.5 and 3 µM). For each experiment, the temperature was shifted to 4°C after 28 min of recording to estimate the degree of protein aggregation after assembly [Weis et al., 2010]. Spectrophotometric curves were corrected so as to reflect only Mt polymerization.

**Statistical analysis**

Differences between datasets were assessed in a nonparametric test (Mann-Whitney-Wilcoxon), with values of P < 0.001 considered significant.

**Online supplemental material**

Fig. S1 shows the screening strategy used in this study. Fig. S2 demonstrates that the SAC is required for viability and to prevent chromosome segregation defects after Ens RNAi. Fig. S3 shows the specificity of the Ensconsin antibody and Ensconsin localization. Table S1 summarizes the phenotypes obtained after RNAi for 18 putative mitotic regulators. Table S2...
The authors declare no competing financial interests.

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