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## Peripheral astral microtubules ensure asymmetric furrow positioning in neural stem cells

Alexandre Thomas, Emmanuel Gallaud, Aude Pascal, Laurence Serre, Isabelle Arnal, Laurent Richard-Parpaillon, Matthew Scott Savoian, Régis Giet

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1 **Peripheral astral microtubules ensure asymmetric furrow positioning in neural stem**  
2 **cells**

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8 Running title: Peripheral astral microtubules recruit a dominant cortical pool of  
9 centralspindlin at the asymmetric division furrow

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13 Alexandre Thomas<sup>1</sup>, Emmanuel Gallaud<sup>1</sup>, Aude Pascal<sup>1</sup>, Laurence Serre<sup>2</sup>, Isabelle Arnal<sup>2</sup>,  
14 Laurent Richard-Parpaillon<sup>1</sup>, Matthew Scott Savoian<sup>3</sup> and Régis Giet<sup>1#</sup>.

15

16 <sup>1</sup>. Univ Rennes, CNRS, IGDR (Institut de Génétique et Développement de Rennes) - UMR  
17 6290, F-35000 Rennes, France

18 <sup>2</sup>. Univ. Grenoble Alpes, Inserm U1216, CEA, CNRS, Grenoble Institut Neurosciences,  
19 GIN, 38000 Grenoble, France.

20 <sup>3</sup>. Massey University, School of Fundamental Sciences, 4410, Palmerston North, New  
21 Zealand

22

23 #. Corresponding author: [regis.giet@univ-rennes1.fr](mailto:regis.giet@univ-rennes1.fr)

24

25 **Lead Contact**

26 Further information and requests for reagents generated in this study should be directed to and  
27 will be fulfilled by the Lead Contact, Régis Giet ([regis.giet@univ-rennes1.fr](mailto:regis.giet@univ-rennes1.fr)).

28

29 **Summary**

30 Neuroblast division is characterized by asymmetric positioning of the cleavage furrow  
31 resulting in a large difference in size between the future daughter cells. In animal cells, furrow  
32 placement and assembly are governed by centralspindlin that accumulates at the equatorial  
33 cell cortex of the future cleavage site and at the spindle midzone. In neuroblasts, these two  
34 centralspindlin populations are spatially and temporally separated. A leading pool is located at  
35 the basal cleavage site and a second pool accumulates at the midzone before travelling to the  
36 cleavage site. The cortical centralspindlin population requires peripheral astral microtubules  
37 and the Chromosome Passenger Complex for efficient recruitment. Loss of this pool does not  
38 prevent cytokinesis but enhances centralspindlin signaling at the midzone leading to  
39 equatorial furrow repositioning and decreased size asymmetry. These data show that basal  
40 furrow positioning in neuroblasts results from a competition between different centralspindlin  
41 pools in which the cortical pool is dominant.

## 42 **Introduction**

43

44 Cytokinesis in somatic cells ensures the equal partitioning of the segregated chromosomes  
45 and is responsible for the division of the mother cell's cytoplasm into two daughters. This  
46 process requires the highly orchestrated assembly and constriction of an acto-myosin  
47 contractile ring, usually at the cell's center. The use of various model systems has clearly  
48 established that the mitotic spindle defines the position of the contractile ring and the resulting  
49 cleavage furrow(von Dassow, 2009, Rappaport, 1971). Two populations of mitotic spindle  
50 microtubules (MTs) have been shown to trigger the assembly of the contractile machinery  
51 during anaphase. The first is a sub-population of astral MTs. These MTs emanate from the  
52 centrosomes to the equatorial cortex where they deliver furrow-inducing signals (Foe and von  
53 Dassow, 2008, Vale et al., 2009, Shannon et al., 2005). The second population, comprises the  
54 spindle midzone, a region of antiparallel MT overlap and interdigitation within the central  
55 spindle that assembles between the decondensing daughter nuclei. In many symmetrically  
56 dividing somatic cell types the relative contribution of these two populations has been  
57 difficult to unambiguously determine due to their close proximity at the cell's equator. Yet,  
58 experiments during the last few decades have led to a proposed common mechanism across  
59 model systems in which the furrow-inducing signals emanate from both cortical proximal  
60 astral MTs and the spindle midzone with each acting in parallel. However, these pathways do  
61 not appear to be equivalent. For instance, if the furrow is initiated at a position distal to the  
62 midzone, it will regress and a new one will be established proximal to it (von Dassow, 2009,  
63 Foe and von Dassow, 2008, Bringmann and Hyman, 2005, Mishima, 2016). Thus, in  
64 equatorially dividing cells, the spindle midzone pathway acts dominantly and can reset furrow  
65 position.

66 Centralspindlin is the main orchestrator of furrowing. This protein complex is a tetramer  
67 composed of two subunits of the Kinesin 6 (Pavarotti-klp in *Drosophila melanogaster*) and  
68 two subunits of the MgcRacGAP (Tumbleweed in *Drosophila melanogaster*). Tumbleweed is  
69 essential for the activation of the Rho-GEF Ect2 (Pebble in *Drosophila melanogaster*). The  
70 formation of Rho-GTP triggers the local activation of Rho Kinase and phosphorylation of  
71 non-muscle Myosin Regulatory Light Chain, an event that stimulates myosin activation and  
72 ultimately drives cytoplasmic cleavage (D'Avino et al., 2015, Glotzer, 2017). While it is well  
73 established that centralspindlin acts along central spindle MTs and accumulates at the cell  
74 cortex equator to promote symmetrical cleavage, far less is known about how this complex  
75 governs asymmetrical divisions. *Drosophila* neural stem cells (Neuroblasts, NBs) are

76 characterized by a biased furrow placement towards the basal region of the cell. Asymmetric  
77 cytokinesis triggers the formation of a large apically positioned cell that retains the NB  
78 identity, and a small basal ganglion mother cell (GMC) that will undergo differentiation  
79 (Knoblich, 2010). Redistribution of myosin immediately after anaphase onset is the important  
80 event that triggers asymmetric cell shape (Cabernard et al., 2010, Connell et al., 2011,  
81 Tsankova et al., 2017). Indeed, apical myosin clearing induces apical cortical expansion. This  
82 is followed by a basal clearing, which similarly induces an expansion of the basal cortex. This  
83 ultimately biases the furrow towards the basal portion of the cell (Cabernard et al.,  
84 2010),(Connell et al., 2011). Previous studies have shown that these two processes are under  
85 the strict control of the NB polarity machinery and influenced by the spindle midzone and the  
86 Chromosome Passenger Complex (CPC) (Roth et al., 2015, Tsankova et al., 2017). To better  
87 understand the mechanism of furrow positioning in asymmetrical cytokinesis, we have  
88 genetically manipulated spindle size and MT dynamics in *Drosophila* NBs. Our data indicate  
89 that the mechanisms dictating asymmetrical daughter cell size are extremely robust and  
90 tolerate increases in spindle length and shape. We report that furrowing initiates in a midzone-  
91 independent manner, at a basal position through the action of a subcortical centralspindlin  
92 pool targeted by peripheral astral microtubules. When these MTs are impaired, centralspindlin  
93 recruitment at the furrow is also affected and becomes abnormally enriched at the midzone  
94 causing repositioning of the cleavage site, thus affecting the size asymmetry of the daughter  
95 cells. Together these results reveal that unlike most systems, in *Drosophila* NBs, which are  
96 characterized by a high level of cell size asymmetry during cell division, a population of  
97 peripheral astral MTs, and not the spindle midzone, defines and maintains asymmetric  
98 cleavage furrow positioning.

99 **Results**

100

101 **Cell size asymmetry is compromised following Ensconsin depletion but not over-**  
102 **expression in NBs**

103 Neuroblasts divide asymmetrically to generate a large self-renewing neuroblast (NB) and a  
104 smaller differentiating ganglion mother cell (GMC, Figure 1 A). We previously showed that  
105 Ensconsin is required for MT polymerization during cell division; consequently *ensc* mutant  
106 spindles are shorter than their wild type (WT) counterparts (Gallaud et al., 2014). To  
107 investigate the possible consequences of a change in spindle length on NB asymmetric cell  
108 division, we first analyzed, by live cell imaging, cell size asymmetry of dividing NBs in  
109 control and *ensc* mutants (Figure 1). We confirmed the previous finding that loss of  
110 Ensconsin triggered a ~10% decrease in mitotic spindle length (Figure 1 B, C). Strikingly, the  
111 *ensc* mutants displayed a small yet statistically significant reduction in the ratio between NB  
112 and GMC diameters indicating a loss of asymmetry (Figure 1 D and J, left). This defect could  
113 either result from the associated change in spindle length or indicate some uncharacterized  
114 function for Ensconsin in asymmetrical size fate determination. To further explore the role of  
115 Ensconsin in MT dynamics *in vitro*, we used TIRF microscopy and recombinant Ensconsin  
116 protein (Figure 1 E). Ensconsin-MBP had a small but significant effect on MT growth rate.  
117 Most striking was the ~50% reduction in the rate of MT shrinkage and the more than 3 times  
118 increase in the rescue frequency compared to controls or MBP alone (Figure 1 F). In line with  
119 these results, over-expression of Ensconsin (Ensc-OE) in NBs lead to elongated spindles that  
120 buckled when reaching the cortex (Figure 1 G, H, S1), consistent with previous work in  
121 symmetrically dividing S2 cells (Gallaud et al., 2014). Despite the increase in MT  
122 polymerization and spindle length, the level of size asymmetry remained unperturbed  
123 following cytokinesis in Ensc-OE NBs (Figure 1 I, and J, right).

124

125 **Enhancement of spindle length through over-expression of Msps or depletion of**  
126 **Kinesin-8 MT depolymerase does not alter cell size asymmetry**

127 To determine if daughter cell size asymmetry is insensitive to stimulation of MT growth, we  
128 quantified size asymmetry following over-expression of the microtubule associated protein  
129 Mini spindles, the fly orthologue of MAP215/ch-TOG, a protein with MT polymerization  
130 properties (Reber et al., 2013, Cullen et al., 1999, Fox et al., 2014). In parallel, we performed  
131 RNAi-mediated depletion of the MT depolymerizing Kinesin-8 fly family member Klp67A.  
132 This kinesin depolymerizes microtubules and its depletion leads to the formation of

133 exceptionally long spindles in *Drosophila* cells (Goshima et al., 2005, Edzuka and Goshima,  
134 2019). Similar to *Ensc*-OE, over-expression of *Msp*s-RFP (*Msp*s-OE) or RNAi-mediated  
135 depletion of *Klp67A* led to the formation of long and bent mitotic spindles (Figure 2 A, B and  
136 D; Figure S1 A; Video S1 and S2). Neither perturbation affected the post-cleavage  
137 asymmetrical cell size (Figure 2 A, C and E). These data suggest that asymmetric cell size  
138 regulation is not sensitive to an increase in MT polymer or spindle length elongation.

139

#### 140 **Spindle shortening through over-expression of Kinesin-8 or -13 MT depolymerases** 141 **decreases cell size asymmetry**

142 To investigate if the size asymmetry reduction observed in *ensc* mutants (Figure 1) was  
143 unique to *Ensc* or rather a common effect of spindle shortening, we induced other  
144 perturbations of MT-polymerization by over-expressing two MT depolymerizing kinesins;  
145 either *Klp10A* which belongs to the Kinesin-13 family (*Klp10A*-OE) or the Kinesin-8  
146 member *Klp67A* (*Klp67A*-OE) (Figure S1 A). Importantly, whereas the depletion of either  
147 causes spindle elongation, their over-expression results in abnormal shortening (Laycock et  
148 al., 2006, Morales-Mulia and Scholey, 2005, Radford et al., 2012, Goshima et al., 2005,  
149 Buster et al., 2007). As predicted both *Klp10A*-OE and *Klp67A*-OE NBs exhibited shorter  
150 spindles although the length reduction was more pronounced in *Klp10A*-OE cells (Figure 2 F,  
151 G, I Video S3). Depending on the spindle's length, it assumed a lesser or greater displacement  
152 relative to the cell center. Interestingly, in these shortened spindle cells, like with *ensc*  
153 mutants, we found that the NB/GMC diameter ratio was significantly impaired indicating that  
154 cell division was more symmetric compared to controls (Figure 2 H and J). Thus, defective  
155 MT polymerization leading to spindle shortening due to loss of *Ensc* function or over-  
156 expression of the Kinesin- 8s and 13s biases asymmetric cell division. An examination of  
157 whole brain phenotypes revealed that *ensc* mutant brain lobes did not undergo any reduction  
158 in NBs. By contrast overexpression of either *Klp67A* (Figure S1 B) or *Klp10A* triggered a  
159 moderate decrease of NBs (Figure S1 C). This diminished NB number was associated with  
160 aneuploidy and polyploidy (Figure S1 D and E). Moreover, we noticed an elevation in cell  
161 cycle duration following *Klp67A*-OE cells (Figure S1 F). Together our data suggest that  
162 spindle shortening after *Klp10A* or *Klp67A* overexpression compromises cell size asymmetry  
163 but also triggers chromosome segregation errors and a loss of NBs.

164

#### 165 **Defective MT growth leads to an apical shift of the basal cleavage furrow after anaphase** 166 **onset**

167 In asymmetrically dividing NBs, it was previously demonstrated that polarity- and spindle-  
168 dependent pathways ensure appropriate myosin distribution to siblings of different sizes  
169 (Cabernard et al., 2010, Roubinet et al., 2017, Tsankova et al., 2017). These two pathways  
170 regulate the time between apical and basal myosin clearing and the subsequent apical and  
171 basal cortical expansions (Figure S2 A). To investigate the behavior of the furrow, we  
172 monitored the dynamics of the regulatory light chain of myosin in live NBs using Sqh-GFP  
173 (Royou et al., 2002). In control cells, we confirmed that Myosin-GFP was uniformly present  
174 at the cell cortex before anaphase (Figure 3 A and Video S4). In *ensc*, Klp67A-OE and  
175 Klp10A-OE NBs, the polarity-dependent apical and basal expansion ratios were similar to  
176 controls (Figure S2 B and C). In metaphase NBs with these same genotypes, the cortical  
177 polarity appeared unaffected, as judged by the presence of strong aPKC and Miranda  
178 crescents at the apical and basal cortices respectively, similar to controls (Figure 2 G). We  
179 also examined the polarity-dependent cleavage furrow-positioning pathway, during anaphase  
180 transition, which results in apical clearing of myosin. We found that the polarity-dependent  
181 apical myosin clearing time was not significantly changed compared to controls (Figure S2  
182 D). We did note a slight dilation in the basal myosin clearing and the time between apical and  
183 basal clearing in *ensc* and Klp10A-OE cells (Figure S2 E and S2 F).

184 We next analyzed furrow positioning through curvature measurements of the cell membrane  
185 ((Tsankova et al., 2017) and Figure 3 B). While the furrow position remained stably placed  
186 from anaphase until cytokinesis in control NBs, we found it shifted significantly towards the  
187 apical side during completion of cell division in *ensc* and Klp67A-OE cells, with a maximal  
188 displacement observed for Klp10A-OE NBs (Figure 3, C Video S5 and S6). While the furrow  
189 width was consistently ~10% of the half-cell cortex length in control NBs as revealed by  
190 Myosin-GFP (Figure 3 D and E), the signal occupied a larger space in *ensc* and Klp67A-OE  
191 NBs with the maximum width of ~25% of the half-cell cortex length observed for Klp10A-  
192 OE cells at comparable time points (Figure 3 A, E, Video S6). From this we conclude that  
193 proper MT growth is required following action of the polarity pathway for maintaining furrow  
194 size and position during asymmetrical cell division.

195

### 196 **Centralspindlin is spatially and temporally regulated as two distinct populations**

197 Our previous perturbations, which interfered with MT dynamics, suggested that a common  
198 mechanism was at play for maintaining the furrow position. In higher eukaryotes, myosin  
199 recruitment and activation at the cleavage furrow is regulated by the highly conserved  
200 centralspindlin complex, a tetramer comprised of a Kinesin-6 family member complexed with



201 Mgc-RacGAP (Pavarotti-klp and Tumbleweed in *Drosophila*, respectively)(D'Avino et al.,  
202 2015, D'Avino et al., 2005, Mishima, 2016). Strikingly, we found that the combination of  
203 Klp10A-OE and a single copy of the *pav*<sup>B200</sup> null allele enhanced the asymmetry defect  
204 observed with Klp10A-OE alone (Figure S2 H). Centralspindlin functionality and targeting to  
205 the membranes is regulated by the chromosomal passenger complex (CPC)-dependent  
206 oligomerization (Basant et al., 2015). We therefore challenged the complex by introducing a  
207 single null allele for its Survivin subunit, *svn*<sup>2180</sup>, and monitored the effects on cell symmetry  
208 in the Klp10A-OE background. We found that Klp10A-OE-dependent size asymmetry defects  
209 were further enhanced when Survivin levels were reduced (Figure S2 H). These results  
210 suggest that the observed asymmetry defects are due, at least in part, to impaired  
211 centralspindlin function.

212 In most eukaryotic cells, the centralspindlin complex is located at the spindle midzone and at  
213 the equatorial cortex. To characterize the furrow mis-positioning that accompanies defective  
214 microtubule growth, we analyzed the spatiotemporal distribution of the motor component of  
215 centralspindlin, Pavarotti-klp, in different experimental backgrounds. We began by examining  
216 GFP-Pav-klp (Minestrini et al., 2003) localization in control NBs. Our time-lapse studies  
217 showed that most of the GFP-Pav-klp was located at the cortex at the cleavage site (Figure 4  
218 A, E and S3 A). Following the onset of furrow ingression, a second pool started to accumulate  
219 into a small and spatially distinct band near the former site occupied by the metaphase  
220 chromosomes at the spindle midzone (Figure 4 A, Figure S3A, and Video S7). The spatial and  
221 temporal separation of the GFP-Pav-klp signals led us to speculate that these were separate  
222 pools of centralspindlin. To confirm this hypothesis, we tracked GFP-Pav-klp in cells lacking  
223 MTs that were forced into anaphase using *Mad2* RNAi to abrogate the spindle assembly  
224 checkpoint (Gallaud et al., 2014). Under these conditions GFP-Pav-klp showed a slight  
225 enrichment at the basal cortex but this pool remained at almost baseline levels compared to  
226 control cells, which showed continuous cortical recruitment of GFP-Pav-klp following  
227 anaphase onset (Figure S3 B and C). When microtubule polymerization was impaired in *ensc*,  
228 Klp10A-OE and Klp67A-OE cells, even if the centralspindlin component GFP-Pav-klp was  
229 initially present at the equatorial cell cortex, it did not become enriched at the cleavage site to  
230 the levels measured in controls (Figure 4 B, C, E red arrowheads, Figure S4 A, see also Video  
231 S8 and S9). Instead, in Klp10A-OE (Video S9), Klp67A-OE but not in *ensc* NBs (Video S8),  
232 GFP-Pav-klp was more abundant at the spindle midzone (Figure 4 B and C, see time 100 s  
233 blue arrows and insets at time 180 s, Figure 4 E and Figure S4 B). Together, these  
234 experiments reveal that centralspindlin exists as two distinct and separable populations, one at

235 the basal cortex and one at the spindle midzone. The decrease of the cortical centralspindlin  
236 pool is always accompanied by a displacement of the cleavage furrow.

237

### 238 **The spatiotemporal regulation of centralspindlin relies on stable peripheral MTs**

239 Fluorescence quantification (Figure 4 F) revealed that compared to controls, Klp10-OE and  
240 Klp67A-OE NBs both displayed a decrease in cortical GFP-Pav-klp signal with a concomitant  
241 increase at the midzone (Figure S4). To further characterize the relationship between cortical  
242 and midzone centralspindlin pools and the role of MT growth in asymmetrical cleavage, we  
243 examined GFP-Pav-klp dynamics in *sas-4<sup>s2214</sup>* mutants, which retain an active polarity  
244 pathway but lack centrosomes and their associated astral MTs (Cabernard et al., 2010, Basto  
245 et al., 2006). In this background, cortical enrichment also appeared diminished relative to the  
246 midzone (Figure 4 D, Figure S4 A and B). An enlarged view of the boxed regions for 180 s  
247 post-anaphase onset highlights this increased centralspindlin recruitment at the spindle  
248 midzone and weaker accumulation at the cell cortex (Figure 4 E). Although *sas-4<sup>s2214</sup>* NBs  
249 exhibited signal enrichment at their midzones, not all cells had a clear cortical reduction  
250 (Figure S4). In addition, *sas-4<sup>s2214</sup>* mutants exhibited both an increased spindle length as well  
251 as a significant overall cell size asymmetry defect (Figure S5 A and B), further supporting the  
252 idea that MT-asters maintain basal furrow position. Similar to previous studies, the cortico-  
253 basal expansion ratios were unchanged in *sas-4<sup>s2214</sup>* NBs (Connell et al., 2011). This indicates  
254 that the centrosome associated MT asters are also important for furrow positioning (Figure S5  
255 D). To confirm the contribution of MT-asters in furrow positioning and maintenance, we  
256 removed them through laser ablation of the apical and basal centrosomes. Each centrosome  
257 was labeled with GFP-tagged Aurora A and apical and basal centrosomes were irradiated by a  
258 multi-photon laser until the signal was no longer detectable. Sample fixation and  
259 immunostaining post-ablation showed the complete disappearance of the Cnn-labeled  
260 centrosome and its associated astral microtubule aster (Figure S5 E, arrowhead). Consistent  
261 with centrosome removal, ablated cells displayed a phenotype virtually identical to *sas-4<sup>s2214</sup>*  
262 mutants: daughter cells exhibited cell size asymmetry after the ensuing cytokinesis (Figure S5  
263 F, G, Video S10, compare left and right). These live cell observations suggested that astral  
264 MTs were essential to cleavage furrow positioning. Accurate quantification of MTs in live  
265 cells was precluded by the lack of spatial resolution in our recordings. We therefore  
266 performed a quantitative analysis of fixed preparations. Although fixed cell approaches can  
267 introduce unintentional bias due to different substage durations that can mask subtle MT  
268 dynamic changes, large-scale trends and differences will be preserved. Detailed morphological

269 examination revealed that in control NBs, bundles of astral MTs were closely apposed to the  
270 cortex at the cleavage furrow. This was not the case with *ensc*, Klp67A-OE or Klp10-OE  
271 NBs, all of which showed decreased MT densities and lacked the presumptive bundles  
272 (Figure S5 H and I). Taken together our data strongly suggest that peripheral astral MTs  
273 originating from the apical and basal centrosomes play a key role in inducing asymmetric cell  
274 division.

275

### 276 **Depletion of the spindle midzone protein Feo does not trigger furrow-positioning defects**

277 The presence of GFP-Pav-klp at the spindle midzone distal to the cleavage site and the  
278 movement of the furrow towards the equator in peripheral MTs-deficient cells prompted us to  
279 further characterize the cleavage site and the midzone in control cells. For this purpose, we  
280 used Fascetto-GFP (the homologue of the mammalian PRC1 protein; Feo-GFP) a marker that  
281 uniquely labels the spindle midzone (Figure 5 A) (Verni et al., 2004, Wang et al., 2015). We  
282 found that in these NBs the metaphase plate was slightly shifted toward the basal side relative  
283 to the cell equator along the apico-basal axis (Figure 5 A, -120 s, blue arrowhead and Figure 5  
284 C, left) but similarly placed to the midzone-defining Feo-GFP signal that appears following  
285 anaphase chromosome segregation (Figure 5 A, time 60 s, Figure 5B and Figure 5 C middle).  
286 This was in contrast to the position of the cleavage furrow (Figure 5 A, time 90, compare  
287 green and red arrowheads; Figure 5 B, 5 C, right), which was always distinct and basally  
288 distal to the midzone (Figure 5 D, Video S11). Kymograph analyses of the spindle midzone  
289 and cell membranes revealed that the midzone moves basally during the ingression of the  
290 furrow until they ultimately consolidate into a single structure (Figure 5 B). These distribution  
291 data raise the possibility that Feo and the spindle midzone may not contribute to furrow  
292 positioning in wild type NBs. We therefore assayed if Feo depletion and spindle midzone  
293 destabilization could impair furrow positioning and asymmetric cell division. We found that  
294 Feo-depleted NBs (Figure 5 E) did not exhibit furrow-positioning defects during anaphase  
295 (Figure 5 F and G) and the final NB/GMC diameter ratio was similar to controls (Figure 5 H).  
296 However, 22% (4/18) of Feo-depleted NBs exhibited late cytokinesis failure (Figure S6 A).  
297 Thus spindle midzone MTs appear to play a key role during the late steps of cytokinesis rather  
298 than in furrow positioning during anaphase. In parallel, we analyzed if the midzone was  
299 reinforced following astral MT depletion (Figure 5 I and J, Figure S6 B). The Feo-GFP signal  
300 intensity at the midzone was enhanced in Klp10A-OE NBs (Figure 5I and S6 C) but remained  
301 unchanged in Klp67-OE and *sas-4<sup>s2214</sup>* NBs. In addition, the signal was decreased in *ensc*  
302 mutant NBs (Figure S6 C). Moreover, the spindle midzone signal was significantly longer in

303 *ensc*, Klp10A-OE, Klp67-OE and *sas-4<sup>s2214</sup>* NBs (Figure S6 D). Interestingly, we also  
304 observed that mitotic spindle length was restored after metaphase and was similar to telophase  
305 controls in Klp10A-OE NBs (Figure S6 E). In these NBs furrow displacement toward the  
306 apical cortex was accompanied by a reduced midzone movement toward the basal cortex  
307 (Figure S6 F). In summary, these data demonstrate that the spindle midzone in NBs occupies  
308 a spatially different position than that of the furrow and its associated cortical MTs. Both of  
309 these MT structures can recruit centralspindlin, however, under normal circumstances in  
310 *Drosophila* NBs it is the cortical pool that dominates to define the cleavage site (Figure S6  
311 G).

## 312 **Discussion**

313

314 Asymmetric cell division is a robust process that ensures that two daughter cells inherit  
315 different fates and sizes. The *Drosophila* NB is a powerful and widely used model system to  
316 study this specialized form of division because of the large number of NBs in the developing  
317 *Drosophila* brain, rapid division time and experimental tractability (Rusan and Peifer, 2007,  
318 Januschke and Gonzalez, 2008, Rebollo et al., 2007). Although these cells are relatively  
319 small, they are highly asymmetrical following cytokinesis allowing accurate measurements  
320 and analyses. In this study, we have challenged asymmetric cell division in this model system  
321 by modifying MT growth dynamics. We were able to increase mitotic spindle length using  
322 over-expression of MT polymerizing MAPs (Msp and Ensconsin), as well as by RNAi-  
323 mediated depletion of Klp67A, a member of the Kinesin-8 family of MT depolymerizing  
324 Kinesins. Despite the presence of long and bent mitotic spindles under these conditions, the  
325 NB cell size ratio remained unchanged relative to control NBs. This reveals that asymmetric  
326 cell division and asymmetric positioning of the cleavage furrow are resistant to an excess of  
327 abnormally long and stable MTs during cell division. By contrast, decreasing MT stability  
328 and shortening the mitotic spindle produced more symmetric cell divisions. This change was  
329 due to an apical shift of the cleavage furrow during its ingression, following apical and basal  
330 cortex expansion. This phenotype was not MAP-dependent and was observed following over-  
331 expression of either Klp10A (Kinesin-13) or Klp67A (Kinesin-8) MT depolymerases and in  
332 *ensc* mutants. Rather the data suggest that spindle size or interference with microtubule  
333 dynamics is responsible for the phenotype. In agreement with this spindle size is restored in  
334 Klp10A-OE telophase cells, which display the shortest spindles at metaphase (Figure S6 E).  
335 Interestingly, *sas-4<sup>s2214</sup>* mutants which are reported to lack functional centrosomes and thus  
336 astral microtubules yielded reduced levels of cell size asymmetry despite harboring longer  
337 metaphase spindles (Basto et al., 2006). This suggests that MT asters and not spindle length  
338 are the key determinant factor for size asymmetry in NBs. Consistent with this, loss of either  
339 apical and basal MT-aster, through targeted laser irradiation and ablation prior anaphase  
340 onset, also reduced sibling cell size asymmetry. Together these results strongly suggest that  
341 astral MTs are required to maintain a cleavage site, which normally favors a basal position in  
342 the fly neuroblast. We propose that a specific population of these astral microtubules, called  
343 peripheral microtubules, are positioned in direct contact with the division furrow and plays a  
344 determining role in maintaining its stable position during anaphase till cytokinesis. For  
345 technical reasons, we were unable to quantify peripheral MTs bundles in live dividing cells.

346 However, fixed cell quantitative, despite a possible bias in the determination of late anaphase  
347 sub stages, support this and revealed a significant decrease in peripheral MTs during in *ensc*,  
348 Klp10A-OE and Klp67A-OE NBs (Figure S5). Our results are in accord with reports  
349 indicating that a subpopulation of these stable astral MTs play a key role in the initiation of  
350 furrowing in symmetrically dividing cells and that in some systems, furrowing can occur  
351 without the presence of a stable central spindle (Murthy and Wadsworth, 2008, Foe and von  
352 Dassow, 2008, Strickland et al., 2005, Canman et al., 2003, Inoue et al., 2004, Bringmann and  
353 Hyman, 2005, Kotynkova et al., 2016, Mishima, 2016). However, in contrast to previous  
354 studies, our data reveals that in asymmetrically dividing control NBs, the astral MT furrowing  
355 pathway dominates over the midzone pathway. Prior investigations indicated that NBs have  
356 two genetically separable pathways to drive cytokinesis. The first, the polarity-dependent  
357 pathway triggers the clearing of apical myosin, resulting in apical cortical expansion.  
358 Interference with this pathway leads to simultaneous apical and basal clearing, symmetrical  
359 cortex expansion, equatorial furrow positioning and to a symmetric division (Cabernard et al.,  
360 2010, Roth et al., 2015, Connell et al., 2011). The second, spindle pathway, is proposed to  
361 rely on the spindle midzone and the chromosomal passenger complex. This triggers the  
362 subsequent basal myosin clearing and basal cortical expansion ((Roth et al., 2015) and Figure  
363 S2 A). Several of the results presented here lead us to propose another additional mechanism  
364 for furrow positioning that would rely on peripheral astral MTs with a minor contribution  
365 from the spindle midzone. This is supported by several of our observations: (i) live cell  
366 imaging and analyses utilizing GFP-Pav-klp as a marker of centralspindlin position revealed  
367 that this master controller of cytokinesis accumulated at the basal cortex throughout the entire  
368 furrow ingression process (this paper and (Cabernard et al., 2010)). (ii) Centralspindlin levels  
369 were low at the midzone during furrow placement and ingression compared to the cortex  
370 (Figure 4). (iii) We consistently found that the midzone, as defined independently using both  
371 GFP-Pav-klp and Feo-GFP, was spatially independent from the furrowing site (Figures 5 B, 6  
372 A). Moreover, inhibition of midzone formation through Feo depletion did not impair furrow  
373 positioning but did interfere with the late stages of cytokinesis. (iv) The midzone consistently  
374 relocated from an initial location to a final position that was coincident with the furrow. The  
375 converse was never observed (Figure 5 B), confirming previous observations made in  
376 embryonic NBs (Kaltschmidt et al., 2000). (v) Finally, genetic or photo-based interference  
377 with centrosomes precluded astral MT formation and interaction with the cortex.  
378 Accordingly, the cortical centralspindlin pool was diminished and NBs exhibited a size  
379 asymmetry defect (Figure 4 and S4).

380 Our localization studies suggest that under normal conditions midzone-associated  
381 centralspindlin does not perform a key role in positioning of the cleavage site and that this  
382 function is served by the more abundant centralspindlin pool associated with the cortex at the  
383 cleavage site. When astral MTs were impaired, centralspindlin enrichment at the furrow was  
384 often diminished, and in some cases accompanied by an increase in the midzone-associated  
385 pool, leading to a decreased midzone/furrow centralspindlin ratio and a reset of the furrowing  
386 toward the equatorial midzone. This indicates that the two populations of centralspindlin are  
387 competent to signal furrowing but that the cortical pool delivered by astral MTs may be  
388 dominant. Thus the spatial localization and the cortical/midzone ratio of centralspindlin are  
389 the pivotal determinants of final furrow position in the *Drosophila* NB. Interestingly, a recent  
390 study has shown that a similar competition between centralspindlin pools also occurs in  
391 human cells, revealing an evolutionary conservation of the mechanism (Adriaans et al., 2019).  
392 As with human cells, we found that the CPC activity seems essential in this regulatory event  
393 (Figure S2 C).

394 In contrast to a recent study in the symmetrically dividing S2 cells, we do not observe GFP-  
395 Pav-klp labeling at the plus ends of astral MTs (Vale et al., 2009, Verma and Maresca, 2019)  
396 even when studied by enhanced resolution imaging methods. Instead, we consistently find  
397 that centralspindlin coats the entire length of astral MTs emanating from both centrosomes,  
398 suggesting that the plus end directed motor activity of Pav-klp is used to bring centralspindlin  
399 to the furrow in *Drosophila* NBs similar to findings in early embryos (Minestrini et al., 2003,  
400 Minestrini et al., 2002). It is therefore likely that centralspindlin, depending on the cell type  
401 utilizes preferentially EB1-mediated MT plus ends or the motor activity of Pav-Klp to reach  
402 the cleavage site.

403 In total our data suggest a model in which competition between different centralspindlin  
404 populations is a key determinant of asymmetric division in *Drosophila* NBs. The consecutive  
405 action of the polarity-dependent cleavage furrow-positioning pathway and the MTs emanating  
406 from the asters serving as centralspindlin delivery arrays are essential in the whole process. In  
407 this system, we propose that the ability of the spindle midzone to define furrow and cleavage  
408 location may only become engaged during late telophase or after subcortical astral MTs are  
409 compromised. Despite their clear role in governing size asymmetry, we have not been able to  
410 induce complete daughter cell size equality through any of a host of MT perturbing  
411 treatments. It is possible that the few MTs that remain after our perturbations are sufficient to  
412 target enough cortical centralspindlin to provide some degree of asymmetry. However,  
413 additional mechanisms, such as MT initial asymmetric midzone position and displacement

414 toward the cleavage site also appears important to secure a minimal level of asymmetry in  
415 these cells (Figure S6 F). Elucidating these systems and their advantages for asymmetrically  
416 dividing stem cells will be important directions for future investigations on tissue  
417 homeostasis.

418  
419

#### 420 Limitation of this study

421 Our study reveals a critical role for astral MTs in maintaining the asymmetric position of the  
422 cleavage site during cytokinesis in neural stem cells. This is essential to preserve an  
423 appropriate Neuroblast/GMC cell size ratio. We find that the perturbation of astral MTs and  
424 the subsequent loss of asymmetry occur in the presence of a timely apical myosin clearing, an  
425 event regulated by the polarity-dependent pathway that controls apical cortical expansion  
426 during anaphase. However, we cannot fully rule out that our observations are completely  
427 polarity independent. Other additional yet uncharacterized polarity-dependent mechanisms  
428 could be involved to contribute to our findings. Indeed, it remains possible that polarity  
429 proteins participate in the regulation of other components including centrosomal or MT-  
430 associated proteins, to control the dynamics of these peripheral astral MTs and ultimately  
431 basal furrow positioning.

432  
433

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447

#### 448 **Author contributions**



449 Conceptualisation, A.T., R.G; Methodology, A.T., E.G., I.A., R.G. Investigation, A.T., A.P.,  
450 E.G., L.S., I.A., R.G.; Writing, A.T and R.G; Review & Editing, A.T., E.G., M.S.S., R.G;  
451 Funding Acquisition, R.G; Resources, R.G; Supervision, L.R-P., R.G.

452

453 **Declaration of interest**

454 The authors have no interest to declare.

455

456 **Main figure titles and legends**

457

458 **Figure 1. Analysis of cell size asymmetry in *ensc* and Ensc-OE NBs**

459 A) Scheme of a NB during cell division during metaphase (left) and telophase (right). Note  
460 that the NB cell division is asymmetric and produces a large NB and a small Ganglion Mother  
461 Cell (GMC) during cytokinesis. B) Selected images of a control NB (top) and an *ensc* mutant  
462 NB (bottom) during cell division. The membranes are displayed in green and the MTs are  
463 displayed in magenta. Time is min:s. Scale bar: 10 $\mu$ m. C) Dot plot showing the mean ( $\pm$  s.d.)  
464 mitotic spindle length/NB diameter ratio in control ( $0.86\pm 0.05$ ,  $n=27$ ) or in *ensc* NBs  
465 ( $0.79\pm 0.06$ ,  $n=23$ ), \*\*\*:  $P<0.0001$  (Mann-Whitney test). D) Dot plot showing the NB mean  
466 ( $\pm$  s.d.) diameter/GMC diameter ratio in control ( $2.29\pm 0.20$ ,  $n=27$ ) or in *ensc* NBs ( $2.16\pm 0.20$ ,  
467  $n=23$ ), \*:  $P<0.05$  (Mann-Whitney test). E) Kymographs showing microtubules assembled  
468 from GMPCPP seeds and 14  $\mu$ M tubulin in absence or in presence of 200 nM of MBP or  
469 MBP-Ensconsin. Horizontal and vertical scale bar are 5 $\mu$ m and 60s respectively. F) Graphs  
470 showing the mean ( $\pm$  s.d.) growth and shrinkage rates and the catastrophe and rescue  
471 frequencies determined from kymographs shown in E. ns: non significant; \*\*\*\*\*:  $P<0.0001$   
472 (Kruskal-Wallis ANOVA followed by post-hoc Dunn's multiple comparison, total number of  
473 growth events = 116, 123 and 107, shrinkage events = 67, 73 and 81, catastrophe events = 94,  
474 96, 83 and rescue events = 3, 3 and 33 for the control, MPB and MPB-ensconsin  
475 respectively). G) Selected images of a control NB (top) and an Ensc-OE NB (bottom) during  
476 cell division. Membranes are displayed in magenta and MTs or Ensconsin are displayed in  
477 green. H) Dot plot showing mean ( $\pm$  s.d.) the mitotic spindle length/NB diameter ratio in  
478 control ( $0.86\pm 0.06$ ,  $n=25$ ) or in Ensc-OE NBs ( $0.95\pm 0.08$ ,  $n=19$ ), \*\*\*\*\*:  $P<0.0001$  (Mann-  
479 Whitney test). I) Dot plot showing the mean ( $\pm$  s.d.) NB diameter/GMC diameter ratio in  
480 control ( $2.36\pm 0.17$ ,  $n=25$ ) or in Ensc-OE NBs ( $2.40\pm 0.19$ ,  $n=19$ ), ns: non-significant (Mann-  
481 Whitney test) J) Summary of NB division in *ensc* (left) or Ensc-OE (right). *ensc* mutant NBs  
482 display shorter spindles and undergo less asymmetric cell division while Ensc-OE NBs,  
483 despite harboring long spindles, divides asymmetrically similar to WT. Time is min:s. See  
484 also Figure S1.

485

486 **Figure 2. Analysis of cell size asymmetry in NB following modification of several MAP**  
487 **protein levels**

488 A) Selected images of control (top), Msp-OE (middle) and *Klp67A* RNAi (bottom) NBs. The  
489 membranes are shown in green, the MTs (top and bottom) and Msp (middle) are shown in

490 magenta. Time is min:s. Scale bar: 10 $\mu$ m. B) Dot plot showing the mitotic spindle length/NB  
 491 diameter ratio ( $\pm$  s.d.) in the NB of control (0.86 $\pm$ 0.06,  $n=18$ ) or in Msps-OE transgenic flies  
 492 (1.03 $\pm$ 0.1,  $n=23$ ), \*\*\*\*:  $P<0.0001$  (Mann-Whitney test). C) Dot plot showing the mean ( $\pm$   
 493 s.d.) NB diameter/GMC diameter ratio in control (2.34 $\pm$ 0.16,  $n=18$ ) or in Msps-OE NBs  
 494 (2.29 $\pm$ 0.28,  $n=21$ ), ns: non-significant (Mann-Whitney test). D) Dot plot showing the mean  
 495 ( $\pm$  s.d.) mitotic spindle length/NB diameter ratio in control NBs (0.83 $\pm$ 0.07,  $n=40$ ) or in  
 496 *Klp67A* RNAi NBs (1.19 $\pm$ 0.15,  $n=30$ ), \*\*\*\*:  $P<0.0001$  (Mann-Whitney test). E) Dot plot  
 497 showing the mean ( $\pm$  s.d.) NB diameter/GMC diameter ratio in control (2.31 $\pm$ 0.16,  $n=40$ ) or  
 498 in *Klp67A* RNAi NBs (2.32 $\pm$ 0.23,  $n=30$ ), ns: non-significant (Mann-Whitney test). F)  
 499 Selected images of control (top), *Klp10A*-OE (middle) and *Klp67A*-OE (bottom) NBs. The  
 500 membranes are shown in green, the MTs are shown in magenta. Scale bar: 10 $\mu$ m. Time is  
 501 min:s. G) Dot plot showing the mean ( $\pm$  s.d.) mitotic spindle length/NB diameter ratio in  
 502 control NBs (0.79 $\pm$ 0.08,  $n=49$ ) or in *Klp10A*-OE NBs (0.63 $\pm$ 0.09,  $n=49$ ), \*\*\*\*:  $P<0.0001$   
 503 (Mann-Whitney test). H) Dot plot showing the NB diameter/GMC diameter ratio ( $\pm$  s.d.) in  
 504 control NBs (2.29 $\pm$ 0.17,  $n=49$ ) or in *Klp10A*-OE NBs (2.00 $\pm$ 0.27,  $n=49$ ), \*\*\*\*:  $P<0.0001$   
 505 (Mann-Whitney test). I) Dot plot showing mean ( $\pm$  s.d.) the mitotic spindle length/NB  
 506 diameter in control NBs (0.83 $\pm$ 0.07,  $n=40$ ) or in *Klp67A*-OE NBs (0.73 $\pm$ 0.08,  $n=48$ ), \*\*\*\*:  
 507  $P<0.0001$  (Mann-Whitney test). J) Dot plot showing the mean ( $\pm$  s.d.) NB diameter/GMC  
 508 diameter ratio in control NBs (2.32 $\pm$ 0.16,  $n=40$ ) or in *Klp67A* RNAi NBs (2.19 $\pm$ 0.18,  $n=48$ ),  
 509 \*\*\*:  $P<0.0001$  (Mann-Whitney test). See also Figure S1.

510

511 **Figure 3. Analysis of myosin dynamics and furrow positioning in *ensc*, *Klp67*-OE and**  
 512 ***Klp10A*-OE**

513 A) Selected images of (from top to bottom) dividing control, *ensc*, *Klp67A*-OE and *Klp10A*-  
 514 OE NBs expressing tubulin (magenta) and myosin regulatory light chain (green and lower  
 515 panels in monochrome) after anaphase onset ( $t=20$ s) till late telophase. Scale bar: 10 $\mu$ m. Time  
 516 is s. B) Scheme showing the possible apical shift between the initial and final furrow  
 517 curvature analysis. C) Dot plot showing mean ( $\pm$  s.d.) of the relative furrow displacement  
 518 between early anaphase and late telophase in control (0.00 $\pm$ 0.04,  $n=18$ ), *ensc* (-0.03 $\pm$ 0.05,  
 519  $n=18$ ), *Klp67A*-OE (-0.05 $\pm$ 0.04,  $n=12$ ), and *Klp10A*-OE NBs (-0.14 $\pm$ 0.04,  $n=22$ ). \*:  $P<0.05$ ,  
 520 \*\*\*:  $P<0.001$ , \*\*\*\*:  $P<0.0001$  (Mann-Whitney test). D) Scheme showing the furrow width  
 521 (red) during mid anaphase. E) Dot plot showing the mean ( $\pm$  s.d.) relative myosin furrow  
 522 width/cell length ratio for control (0.08 $\pm$ 0.02,  $n=18$ ), *ensc* (0.14 $\pm$ 0.07,  $n=17$ ), *Klp67A*-OE

523 (0.13±0.05, n=12), and Klp10A-OE NBs (0.25±0.11, n=23), \*:  $P<0.05$ , \*\*\*:  $P<0.001$ , \*\*\*\*:  $P<0.0001$  (Mann-Whitney test). See also Figure S2.

525

526 **Figure 4. Analysis of centralspindlin localization and dynamics in control, *ensc*, Klp10A-**  
527 **OE and *sas-4<sup>s2214</sup>* NBs**

528 A) Selected images of dividing control expressing tubulin (magenta) and GFP-Pav-klp (green  
529 and lower panels in monochrome) from anaphase onset till late telophase (top left). B) *ensc*  
530 NB. C) Klp10A-OE NB. D) *sas-4<sup>s2214</sup>* NBs (bottom). Scale bar: 10µm. Time is s. E) Higher  
531 magnification view of the selected control *ensc*, Klp10A-OE and *sas-4<sup>s2214</sup>* telophase NBs  
532 (from panels in A-D) showing GFP-Pav-klp localization at the cleavage site. See the strong  
533 signal at the cell cortex (red arrowheads) and the weak signal at the presumptive spindle  
534 midzone away from the cleavage site, toward the apical side blue (blue arrows). F) Scheme of  
535 the cleavage site showing the cortical and midzone centralspindlin pools. G) Dot plot showing  
536 the mean (± s.d.) relative cortical/midzone GFP intensity ratio for control (2.21±0.89, n=15),  
537 *ensc* (1.57±0.70, n=11), Klp10A-OE (1.09±0.61, n=10), *sas-4<sup>s2214</sup>* (0.84±0.35, n=10), control  
538 (3.24±1.40, n=7) and Klp67A-OE NBs (1.77±0.55, n=11). \*:  $P<0.05$ , \*\*\*:  $P<0.001$ , \*\*\*\*:  $P<0.0001$  (Mann-Whitney test). See also Figures S3, S4 and S5.

540

541 **Figure 5. Analysis of the spindle midzone and the cleavage furrow position in brain NBs**  
542 **during cell division**

543 A) Selected images of a WT NB expressing Feo-GFP (green and lower panels in  
544 monochrome), MTs (magenta), and membranes during cell division (green and lower panels  
545 in monochrome). The metaphase plate is indicated by a blue arrowhead (-120s). Red  
546 arrowheads indicate the spindle midzone and the furrow is indicated by green arrowheads (60-  
547 300s). Scale bar: 10 µm. Time is s. B) Kymograph showing the localization of the spindle  
548 midzone and the cell contours during the time course of the NB cell division shown in panel  
549 A, along the apico-basal axis. Vertical scale bar: 5 µm. C) Dot plot showing the mean (± s.d.)  
550 relative metaphase plate position along the apico-basal cortex 90 s after anaphase onset (left:  
551 0.56±0.02 n=21) the mean (± s.d.) relative spindle midzone position along the apico-basal  
552 cortex 90 s after anaphase onset (middle: 0.59±0.02, n=23) and the mean (± s.d.) relative  
553 furrow position along the apico-basal cortex 90 s after anaphase onset (right: 0.72±0.13,  
554 n=23). D) Dot Plot showing the mean (± s.d.) relative distance between the furrow and the  
555 spindle midzone 90 s after anaphase onset (0.13 ± 0.02, n=23). E) Western blot of Feo (top)  
556 and actin (bottom) protein levels in control or *Feo* RNAi brains. F) Control (top) or *Feo*

557 RNAi NBs expressing PH-PLC $\delta$ -GFP (green and bottom panels in monochrome) and  
558 mCherry-tubulin (magenta). G) Dot plot showing the mean ( $\pm$  s.d.) relative midzone position  
559 for control ( $0.69\pm 0.03$ ,  $n=16$ ) and *Feo* RNAi NBs ( $0.69\pm 0.02$ ,  $n=18$ ). Ns: not significant  
560 unpaired T test. H) Dot plot showing the mean ( $\pm$  s.d.) NB/GMC size ratio for control  
561 ( $2.36\pm 0.12$ ,  $n=16$ ) and *Feo* RNAi NBs ( $2.40\pm 0.15$ ,  $n=14$ ). Note late cytokinesis failed in 4/18  
562 NBs (22%). Ns: not significant (Unpaired T test). I) Selected frames of a control (top) or a  
563 Klp10A-OE NB expressing *Feo*-GFP (green and bottom panels in monochrome) and  
564 mCherry-tubulin (magenta) after anaphase onset (0 s). Arrowheads indicate midzone-  
565 associated *Feo*-GFP. Scale bar: 10  $\mu$ m. Time is s. J) Dot plot showing the mean ( $\pm$  s.d.) time  
566 (s) between *Feo*-GFP detection on the midzone and basal myosin clearing in control  
567 ( $59.4\pm 15.4$ ,  $n=18$ ) and Klp10A-OE ( $108.7\pm 40.5$ ,  $n=15$ ) Nbs. \*\*\*\*:  $P<0.0001$  (Mann-Whitney  
568 test). See also Figure S6.  
569

570 **STAR Methods**

571

572 **RESOURCE AVAILABILITY**

573 **Lead Contact**

574 Further information and requests for reagents generated in this study should be directed to and  
575 will be fulfilled by the Lead Contact, Régis Giet (regis.giet@univ-rennes1.fr).

576 **Materials Availability**

577 All unique/stable reagents generated in this study are available from the lead contact without  
578 restriction.

579 **Data and Code Availability**

580 § All data reported in this paper will be shared by the lead contact upon request.

581 § This paper study does not report original code.

582 § Any additional information required to reanalyze the data reported in this work paper is  
583 available from the Lead Contact upon request.

584

585 **EXPERIMENTAL MODELS AND SUBJECT DETAILS**

586 **Fly strains**

587 All flies were maintained under standard conditions at 25°C. The *ensconsin* mutant fly stocks  
588 *enscAnull* and *enscAN*, referred to *ensc* flies, were characterized previously (Gallaud et al.,  
589 2014). UAS-Ensconsin-Venus transgenic flies have been characterized in a previous study  
590 (Metivier et al., 2019). UAS-Msps-RFP overexpressing flies were obtained from BestGene  
591 (USA) following P-element mediated transformation. UAS-Klp10A flies were supplied by C.  
592 Dahmann (Max Planck Institute, Germany) (Widmann and Dahmann, 2009). UAS-Klp67A  
593 (ID # F001232) stock was obtained from FlyORF (Bischof et al., 2013). UAS-Klp67A-RNAi  
594 (VDRC ID 52105) and UAS-Mad2-RNAi (VDRC ID 106003) transgenic fly lines were  
595 obtained from the Vienna Drosophila RNAi Center (Dietzl et al., 2007). Sqh-GFP (Royou et  
596 al., 2002), UAS-GFP-Pav-klp (Minestrini et al., 2003) and Ubiquitin-β-tub-GFP expressing  
597 flies (Inoue et al., 2004, Minestrini et al., 2003) were supplied by R. Karess (Institut Jacques  
598 Monod, France) and by D. Glover (University of Cambridge, UK), respectively. The Pavarotti  
599 mutant *pav<sup>B200</sup>* flies were obtained from E. Montebault (Institut Européen de Chimie et  
600 Biologie, France) (Adams et al., 1998) and the Survivin mutant allele *svn<sup>2180</sup>* flies were  
601 courtesy of Jean-René Hyunh (College de France, France) (Mathieu et al., 2013). Flies  
602 expressing the membrane-localized PH-PLCδ-GFP and PH-PLCδ-RFP proteins were  
603 provided by A. Guichet (Institut Jacques Monod, France) (Claret et al., 2014, Gervais et al.,

604 2008). RFP-Tubulin flies were provided by R. Basto (Institut Curie, France). The GFP-AurA  
605 expressing fly stock was described previously (Caous et al., 2015). The following stocks were  
606 obtained from the Bloomington Stock Center: Feo-GFP expressed under the ubiquitin  
607 promoter (BDSC 59273, (Wang et al., 2015)), *sas-4<sup>s2214</sup>* mutant (BDSC 12119, (Basto et al.,  
608 2006)), 69B-Gal4 (BDSC 1774), Insc-Gal4 (BDSC 8751), UAS-mCherry- $\alpha$ -tubulin (BDSC  
609 25774 and BDSC 25773). The *Feo* RNAi stock was from the Vienna Drosophila RNAi  
610 Center (VDRC 107824). The 69B-Gal4 fly stock was used to drive over-expression in the fly  
611 CNS for the following UAS regulated transgenes: Klp67A, Msps, GFP-Pav-Klp together with  
612 *Mad2* RNAi and UAS-mCherry at 25°C for 3 days and 2 days at 29°C for *Feo* RNAi. The  
613 Insc-Gal4 strain was used to drive over-expression of Ensconsin, Klp10A, UAS-GFP-Pav-klp  
614 and mCherry- $\alpha$ -tubulin transgenes in the central brain at 25°C.

615

## 616 **METHOD DETAILS**

### 617 **Molecular biology**

618 Msps cDNA was provided by G. Rogers (University of Arizona, USA), amplified by PCR  
619 and inserted into pDONR221 (Life Technologies) to generate the pDONR221-Msps entry  
620 clone. pENTR-Ensc has been previously described (Gallaud et al., 2014). pDONR221-Msps  
621 entry clone was subsequently recombined into pTWR (Carnegie Institute, USA) using the  
622 Gateway recombination cloning technology (Life Technologies) to generate a construct  
623 allowing the expression of Msps-RFP fusion proteins under the control of the GAL4  
624 protein. pENTR-Ensc was recombined into pDEST-MBP (a gift from H. Ohkura, University  
625 of Edinburgh, UK) to allow the expression of a recombinant Ensconsin protein with a C-  
626 terminal Maltose Binding Protein tag.

627

628

### 629 **Production of recombinant proteins**

630 MBP and Ensconsin-MBP were induced in *E. coli*, for 4 h at 25°C. The proteins were purified  
631 on amylose columns as described by the manufacturer (BioLabs) and stored in small aliquots  
632 at -80°C.

633

### 634 **TIRF microscopy and analysis of MT dynamics**

635 Tubulin was purified from bovine brain and fluorescently labeled with ATTO 488 and ATTO  
636 565 or biotinylated as described before (Hyman et al., 1991, Ramirez-Rios et al., 2017).

637 Briefly, microtubule seeds were prepared from biotinylated and ATTO-565-labeled tubulin in  
638 the presence of Guanosine-5'-[( $\alpha,\beta$ )-methylene]triphosphate (GMPCPP) in BRB80 buffer (80  
639 mM Pipes, 1 mM EGTA, 1 mM MgCl<sub>2</sub>, pH 6.74) (Ramirez-Rios et al., 2017). Flow chambers  
640 were prepared with functionalized silane-PEG-biotin coverslips and silane-PEG glass slides,  
641 as previously described (Ramirez-Rios et al., 2017). The chamber was successively perfused  
642 at room temperature with neutravidin (25  $\mu$ g/ml in 1% BSA in BRB80), PLL-g-PEG (2 kD,  
643 0.1 mg/ml in 10 mM Hepes, pH 7.4), BSA (1% in BRB80 buffer) and microtubule seeds. The  
644 following assembly mixture was then injected: 14  $\mu$ M tubulin (containing 15 % ATTO-488-  
645 labeled tubulin) without or with 200 nM MBP or MBP-Enscosin in TIRF assay buffer (4 nM  
646 DTT, 50 mM KCl, 1% BSA, 1 mg/mL glucose, 70  $\mu$ g/mL catalase, 580  $\mu$ g/mL glucose  
647 oxidase, 0.05% methylcellulose (4000 centipoise) in BRB80). Time-lapse images were  
648 recorded at 35°C at a rate of one frame per 5 seconds on an inverted Eclipse Ti Nikon  
649 microscope equipped with an Apochromat 60X1.49 N.A oil immersion objective, an iLas<sup>2</sup>  
650 TIRF system (Roper Scientific), and a cooled charge-coupled device camera (EMCCD  
651 Evolve 512, Photometrics) controlled by MetaMorph 7.7.5 software.

652 Microtubule dynamic parameters were analyzed in Image J on kymographs obtained using an  
653 in-house KymoTool macro (available upon request to eric.denarier@univ-grenoble-alpes.fr).  
654 Growth and shrinkage rates were determined from the slopes of microtubule growth and  
655 shrinkage phases. The catastrophe and rescue frequencies were calculated by dividing the  
656 number of events per microtubule by the time spent in growing and shrinking states,  
657 respectively.

658

### 659 **Antibodies and Western blotting**

660 The following antibodies and concentrations were used in this study: polyclonal rabbit anti-  
661 Msps (1:5000) provided by J. Raff (Lee et al., 2001), polyclonal rabbit anti-Klp67A (1:500)  
662 supplied by G. Goshima (Goshima and Vale, 2005), polyclonal rabbit anti-Feo antibody  
663 (1:2000) was provided by J. Scholey (Wang et al., 2015), polyclonal rabbit anti-Klp10A  
664 (1:1000) was courtesy of G. Rogers (Mennella et al., 2005) and rabbit anti-Myosin (1:2000)  
665 was provided by R. Karess (Jordan and Karess, 1997). The anti-Enscosin antibody raised  
666 against the Kinesin binding domain has been previously described (Gallaud et al., 2014).  
667 Rabbit anti-PKC $\zeta$  (C-20, 1:200) and anti-actin polyclonal antibodies (sc-1616, 1:5000) were  
668 obtained from Santa Cruz Technology. Monoclonal mouse anti-alpha Tubulin (clone DM1A,  
669 T2199; 1:500) and rabbit polyclonal anti-phosphorylated histone H3 (Ser10) (06570, 1:500)  
670 antibodies were obtained from Millipore. Monoclonal rat anti-Miranda antibody (ab197788,



671 1:1000) was obtained from Abcam. Rat anti-Deapan (AB195173, 1:100) and mouse anti-  
672 Propero (MR1A, 1:500) antibodies were from DSHB. Secondary antibodies were labeled with  
673 either Alexa Fluor-conjugated (1:1000) or peroxidase-conjugated secondary antibodies  
674 (1:5000), each obtained from Life Technologies. For Western Blotting ECL reagents were  
675 purchased from ThermoFisher.

676

### 677 **Live cell microscopy**

678 Third-instar larval brains were dissected in Schneider's *Drosophila* medium supplemented  
679 with 10% FCS. Isolated brains were loaded and mounted on stainless steel slides, and the  
680 preparations were sealed with mineral oil (Sigma-Aldrich) as previously described (Gallaud et  
681 al., 2014). For MT depolymerization experiments, larval brains were incubated during 30 min  
682 in the above medium supplemented with colchicine at a final concentration of 15  $\mu$ M. After  
683 incubation, brains were mounted and processed for live cell imaging.

684 Images were acquired at 25°C using a CSU-X1 spinning-disk system mounted on an inverted  
685 microscope (Elipse Ti; Nikon) equipped with a 60X 1.4 NA objective. At 20, 30 or 60 s  
686 intervals 10 z-steps were acquired with 1 $\mu$ m intervals. Fluorescent protein probes were  
687 excited with 488nm or 561nm laser light and the images were captured using a sCMOS  
688 ORCA-Flash4.0 (Hamamatsu) camera. Recordings were controlled using MetaMorph  
689 acquisition software. Alternatively, images were acquired with a spinning disk system  
690 consisting of a DMI8 microscope (Leica) equipped with a 63X (1.4 N.A.) oil objective, a  
691 CSU-X1 spinning disk unit (Yokogawa) and an Evolve EMCCD camera (Photometrics). The  
692 microscope was controlled by the Inscoper Imaging Suite and the dedicated software  
693 (Inscoper). Data were processed in ImageJ and viewed as maximum-intensity projections  
694 prior to analysis or figure preparation.

695

### 696 **Photo-ablation experiments**

697 Photo-ablations were performed with a Mai-Tai two-photon infrared laser (Spectra Physics)  
698 attached to a Leica SP5 confocal microscope equipped with a 60X 1.3 NA objective with the  
699 stage maintained at 25°C. Z-series consisting of 10, 1 $\mu$ m steps were acquired before and after  
700 the photo-ablation at 30 s intervals. Photo-ablation was performed on the basal and apical  
701 centrosomes using flies expressing GFP-H2A, PH-PLC $\delta$ -GFP and Aurora A-GFP. Anaphase  
702 onset was identified as the first signs of sister chromatid separation. The rapid (<10 s,  
703 (Berdnik and Knoblich, 2002)) recovery of Aurora A-GFP was used to differentiate between  
704 centrosome photo bleaching and photo-ablation. Photo-ablation efficacy was confirmed using

705 cells co-expressing RFP-tubulin. Cells that did not recovery the Aurora A-GFP signal within  
706 30 s post-irradiation also displayed a loss of the MT-aster as reveled in live and fixed cell  
707 analyses.

708

### 709 **Live cell imaging analysis**

710 Measurements of fluorescence intensities, distances, mitotic spindle lengths and diameters of  
711 NB and GMC cells were performed with ImageJ software (Rueden et al., 2017). The Sqh-  
712 GFP analyses were done on the maximum projection of two optical sections (1  $\mu\text{m}$ ). The  
713 cortical GFP-Pav-klp intensity analyses on the furrow during anaphase were calculated as the  
714 mean normalized intensity signal between the two sides of the furrow on two optical sections  
715 (1  $\mu\text{m}$ ). Cell cycle duration was calculated as the time between two consecutive NEBD  
716 events. The polarity-dependent apical and basal clearings were calculated, as the time point  
717 after anaphase onset, when myosin disappeared from the apical and basal cortices respectively  
718 (Connell et al., 2011),(Roubinet et al., 2017). NB cortex curvature analyses were performed  
719 according to previously defined methods (Tsankova et al., 2017). The furrow shift was  
720 determined as the distance between the first ingression site and final cleavage site. To  
721 quantify the Myosin-GFP furrows width; a segmented line was drawn along the NB half-cell  
722 cortex during anaphase and the GFP intensity profiles were quantified along this line using  
723 ImageJ. The furrow width was measured as the relative half-cell cortex length containing 60%  
724 of the maximum Sqh-GFP signal intensity. Cortical expansion analyses were performed as  
725 described previously (Connell et al., 2011). Detailed analyses are illustrated in Figure S2A.

726

### 727 **Immunofluorescence analysis**

728 Larval brains from each genotype were processed for immunofluorescence studies as  
729 described previously (Gallaud et al., 2014). Briefly, wandering third instar larval brains were  
730 dissected in testis buffer (TB: 183 mM KCL, 47 mM NaCl, 10 MM Tris, and 1 mM EDTA,  
731 pH 6.8) and brains were fixed for 20 minutes at 25°C in TBF (TB supplemented with 10%  
732 formaldehyde, and 0.01% Triton X-100). Brains were then washed twice in PBS for 15  
733 minutes, and twice in PBS Triton X-100 0.1% for 15 minutes. The brains were first incubated  
734 for 60 minutes at 25°C in PBSTB (1% BSA), before incubation with secondary antibodies.  
735 The samples were observed with a SP5 confocal microscope (Leica) equipped with a 63X  
736 1.4NA objective lens. Images are maximum intensity projections consisting of 4 optical  
737 sections acquired at 0.5  $\mu\text{m}$  intervals. The central brain NB number was calculated as the

738 number of deadpan positive cells and the percentages of euploid, aneuploid and polyploid  
739 cells were determined on brain squashed preparation as described before (Caous et al., 2015).

740

## 741 **QUANTIFICATION AND STATISTICAL ANALYSES**

### 742 **Quantification of peripheral MTs in fixed NBs during mid anaphase**

743 Z-series were acquired every 0.2  $\mu\text{m}$  using a LSM 880 confocal microscope with Airyscan  
744 (Zeiss) for telophase NBs. Images were then processed with the Zen software. Images were  
745 analyzed with ImageJ as maximum intensity projections (0.8  $\mu\text{m}$ ) consisting of 5 optical (0.2  
746  $\mu\text{m}$ ) sections in the plane of the furrow.

747

### 748 **Statistical analysis**

749 Differences between datasets were assessed with Prism 7.0a software (GraphPad), either by  
750 non-parametric tests (Mann-Whitney) or parametric tests (Unpaired T). Non-significance (ns)  
751 threshold was when  $P > 0.05$ . Statistical details can be found in the figure legend, with each  
752 “n” representing a distinct neuroblast.

753

754

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933

934

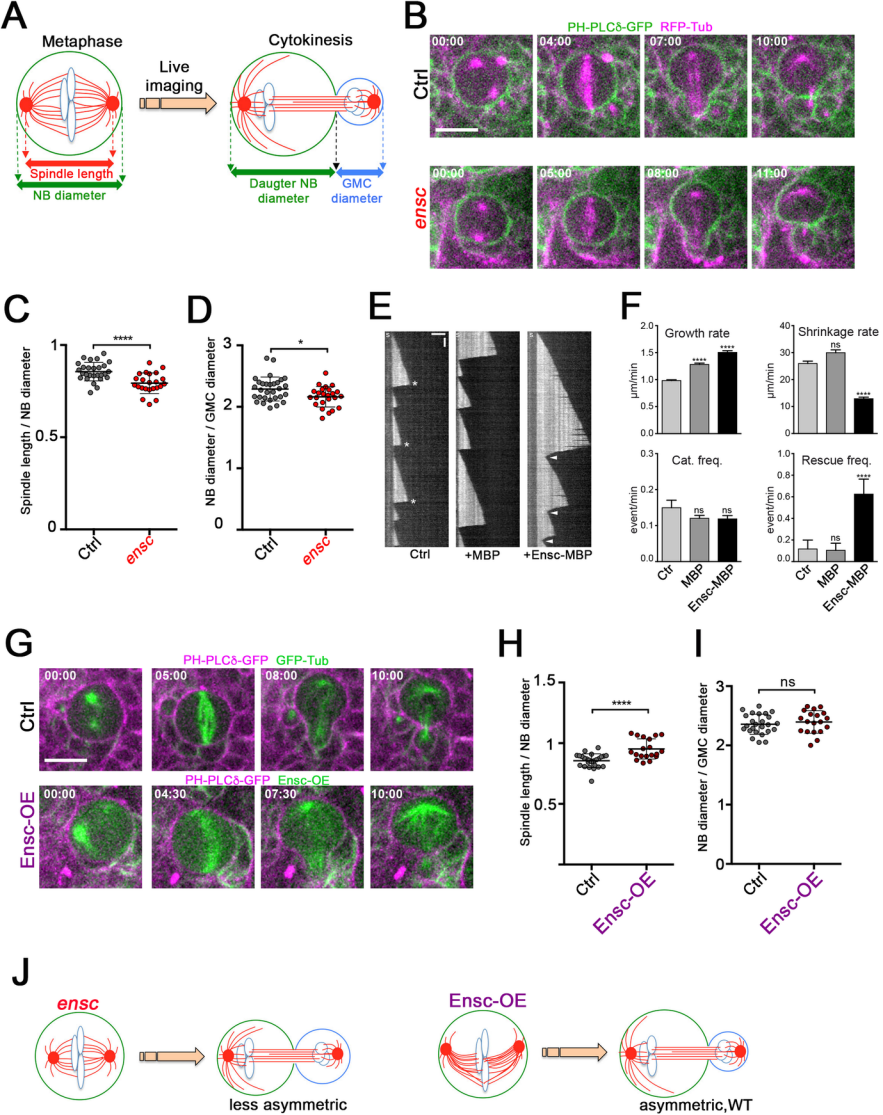


Figure 1



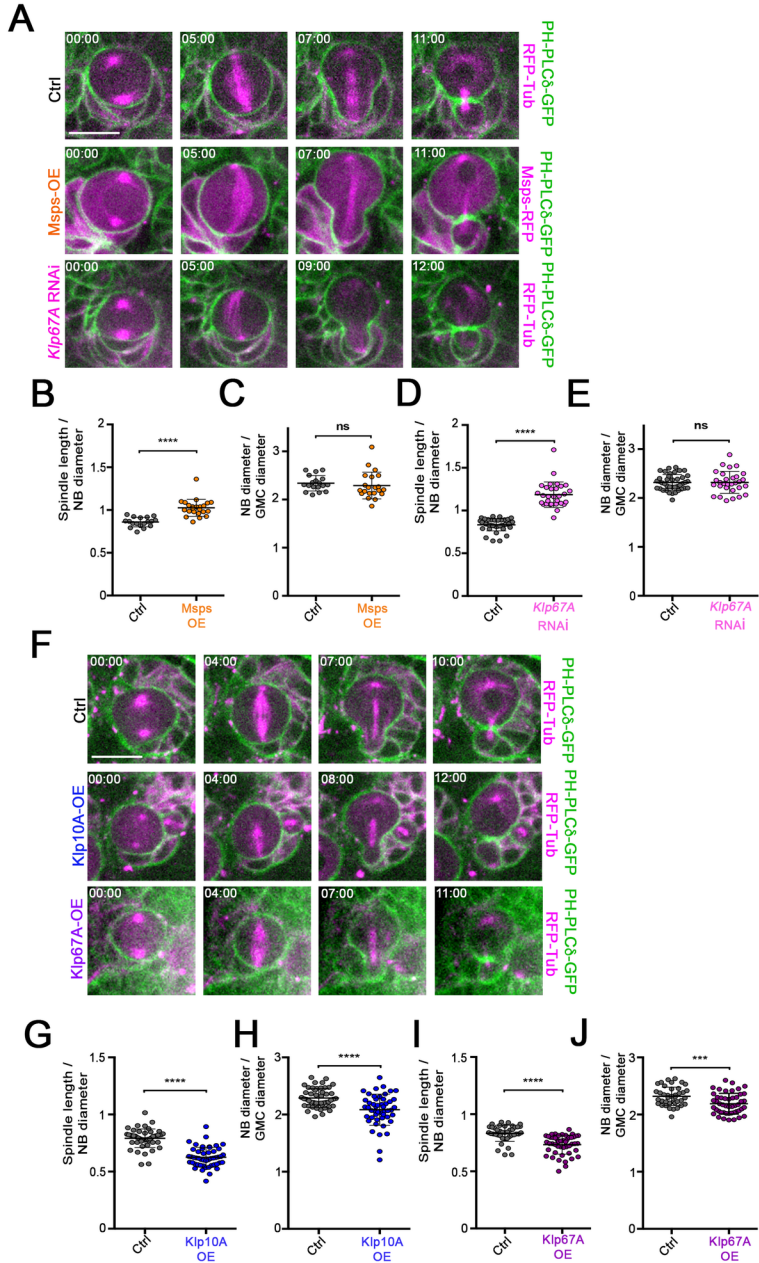


Figure 2

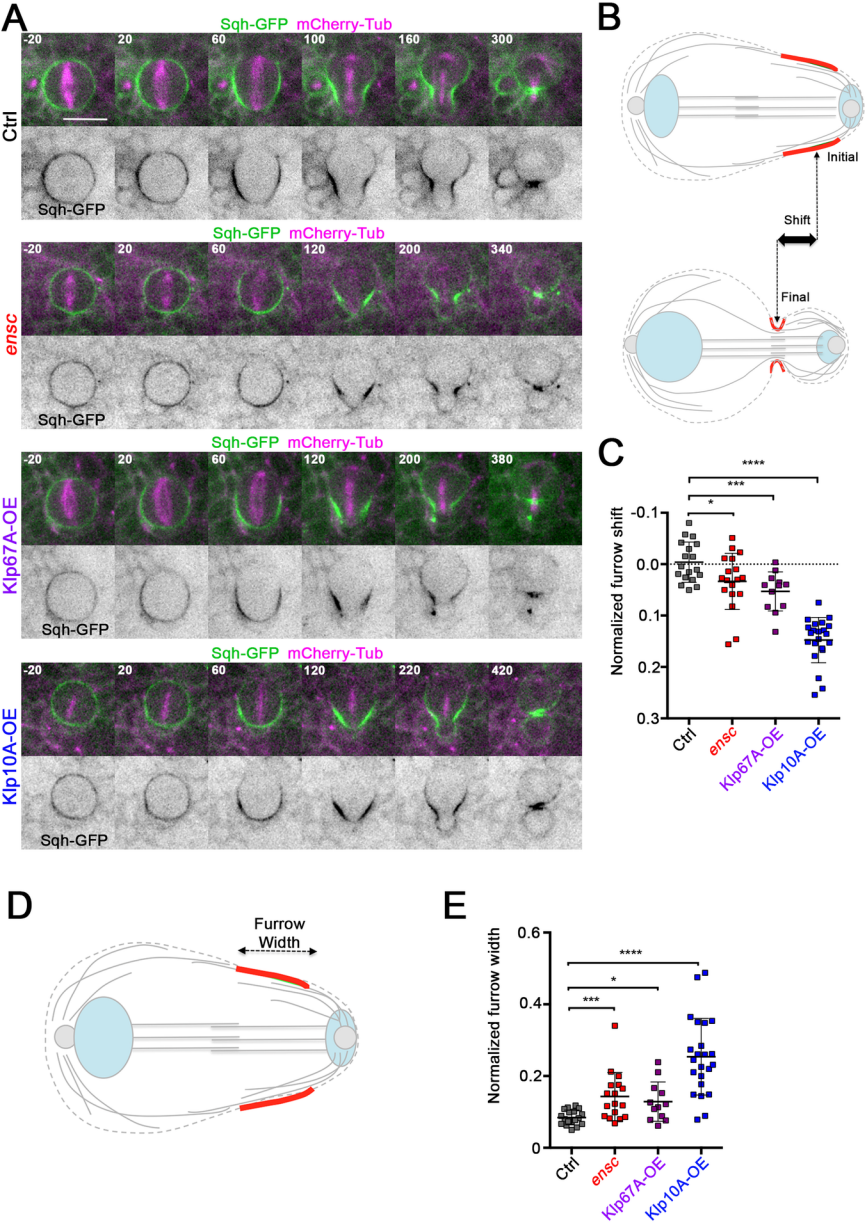


Figure 3

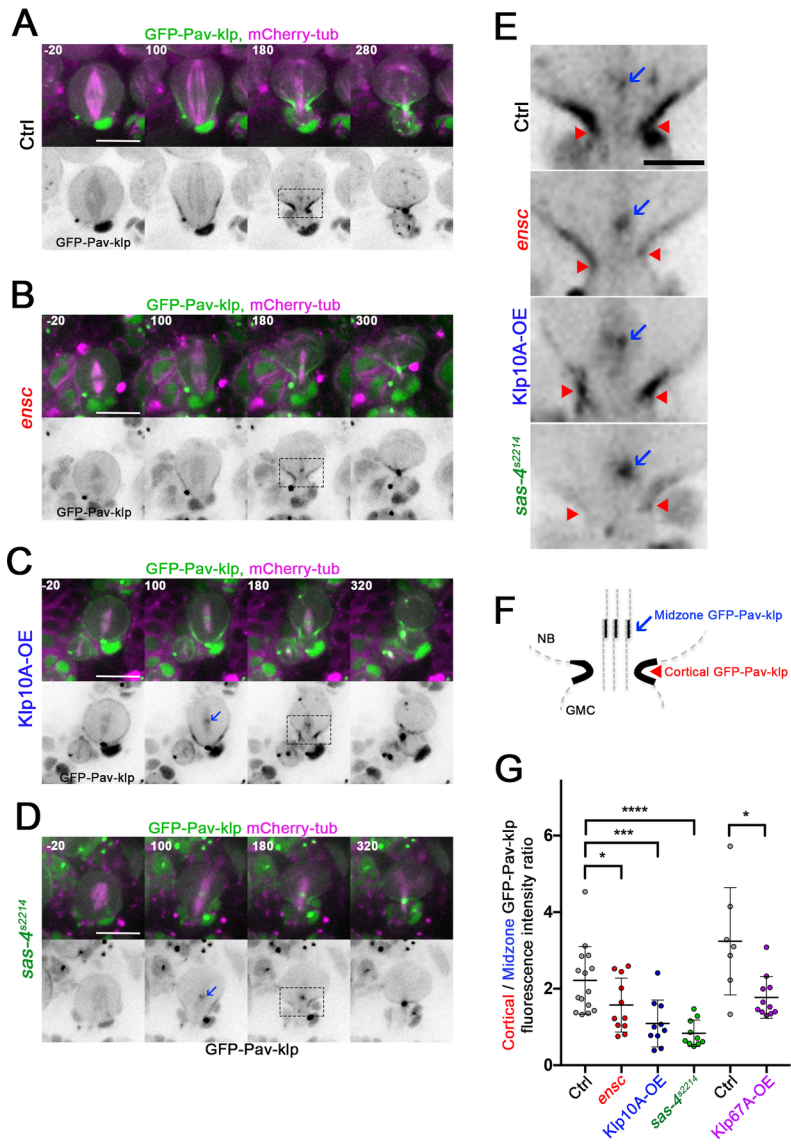


Figure 5

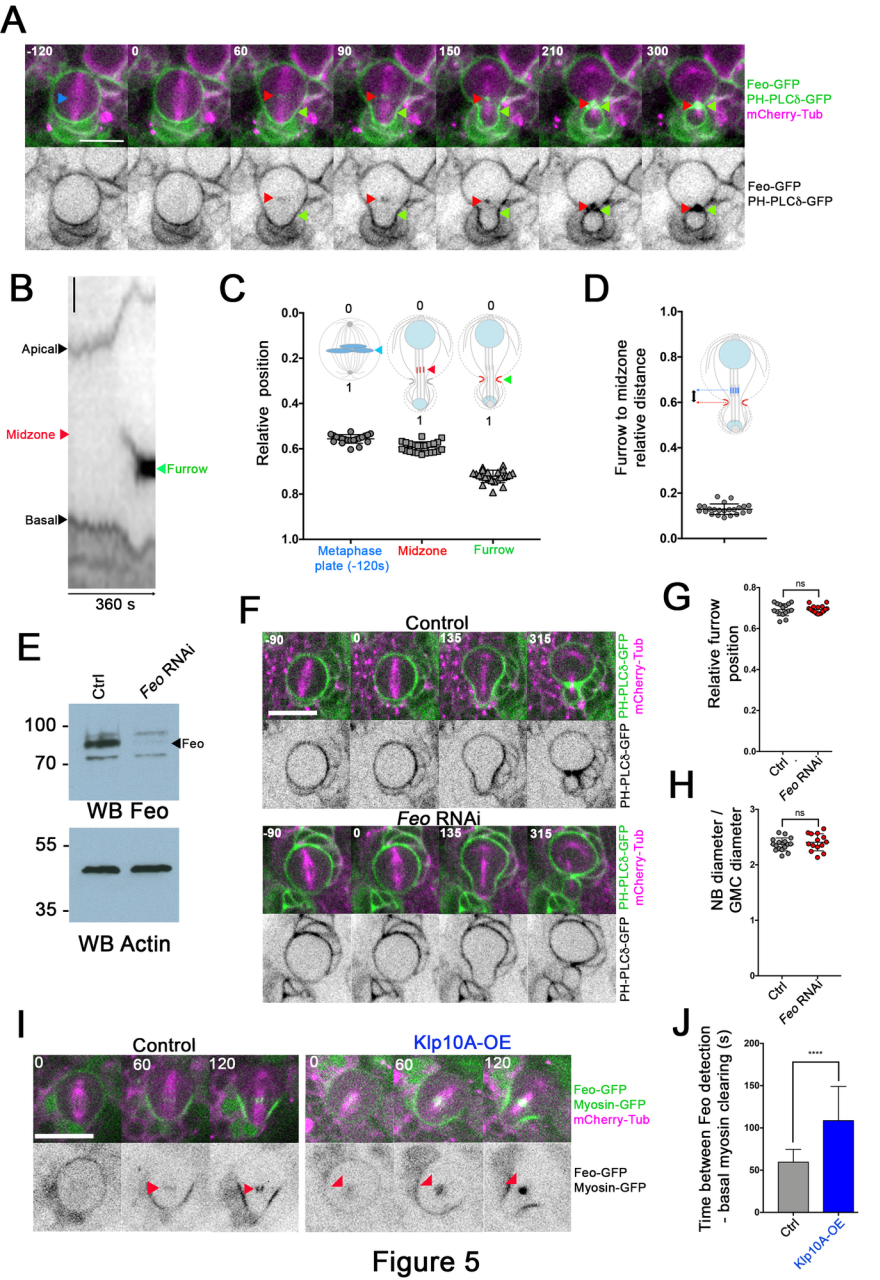
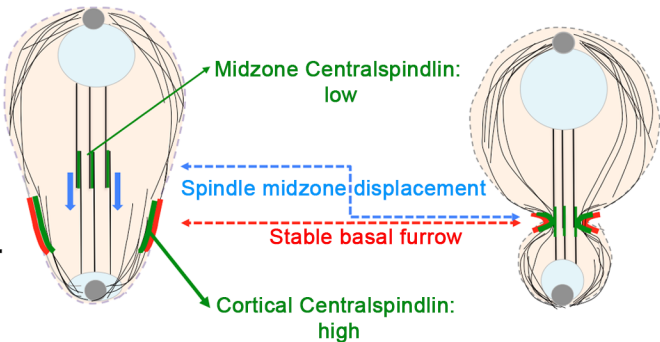


Figure 5

## Peripheral astral MTs



## Defective astral MTs

