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Peripheral astral microtubules ensure asymmetric furrow positioning in neural stem cells

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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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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).

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*^{B200} 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*²¹⁸⁰, 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^{s2214}* 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^{s2214}* NBs
249 exhibited signal enrichment at their midzones, not all cells had a clear cortical reduction
250 (Figure S4). In addition, *sas-4^{s2214}* 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^{s2214}* 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^{s2214}*
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^{s2214}* 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^{s2214}* 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 (Mps and Enscosin), 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^{s2214}* 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 μ m. C) Dot plot showing the mean (\pm s.d.)
464 mitotic spindle length/NB diameter ratio in control (0.86 ± 0.05 , $n=27$) or in *ensc* NBs
465 (0.79 ± 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 ± 0.20 , $n=27$) or in *ensc* NBs (2.16 ± 0.20 ,
467 $n=23$), *: $P<0.05$ (Mann-Whitney test). E) Kymographs showing microtubules assembled
468 from GMPCPP seeds and 14 μ M tubulin in absence or in presence of 200 nM of MBP or
469 MBP-Ensconsin. Horizontal and vertical scale bar are 5 μ 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 ± 0.06 , $n=25$) or in Ensc-OE NBs (0.95 ± 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 ± 0.17 , $n=25$) or in Ensc-OE NBs (2.40 ± 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 μ 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 μ 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 μ 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^{s2214}* 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^{s2214}* NBs (bottom). Scale bar: 10µm. Time is s. E) Higher
531 magnification view of the selected control *ensc*, Klp10A-OE and *sas-4^{s2214}* 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^{s2214}* (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 δ -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 ± 0.03 , $n=16$) and *Feo* RNAi NBs (0.69 ± 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 ± 0.12 , $n=16$) and *Feo* RNAi NBs (2.40 ± 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 μ 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 ± 15.4 , $n=18$) and Klp10A-OE (108.7 ± 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^{B200}* flies were obtained from E. Montebault (Institut Européen de Chimie et
600 Biologie, France) (Adams et al., 1998) and the Survivin mutant allele *svn²¹⁸⁰* 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 Currie, 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^{s2214}* mutant (BDSC 12119, (Basto et al.,
608 2006)), 69B-Gal4 (BDSC 1774), Insc-Gal4 (BDSC 8751), UAS-mCherry- α -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- α -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'-[(α,β)-methylene]triphosphate (GMPCPP) in BRB80 buffer (80
639 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, 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 μ 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 μ 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 μ g/mL catalase, 580 μ 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 iLas²
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 ζ (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 μ 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 μ 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 μ 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 δ -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 μm). 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 μm). 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 μm 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 μm 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 μm) consisting of 5 optical (0.2
746 μm) 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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934

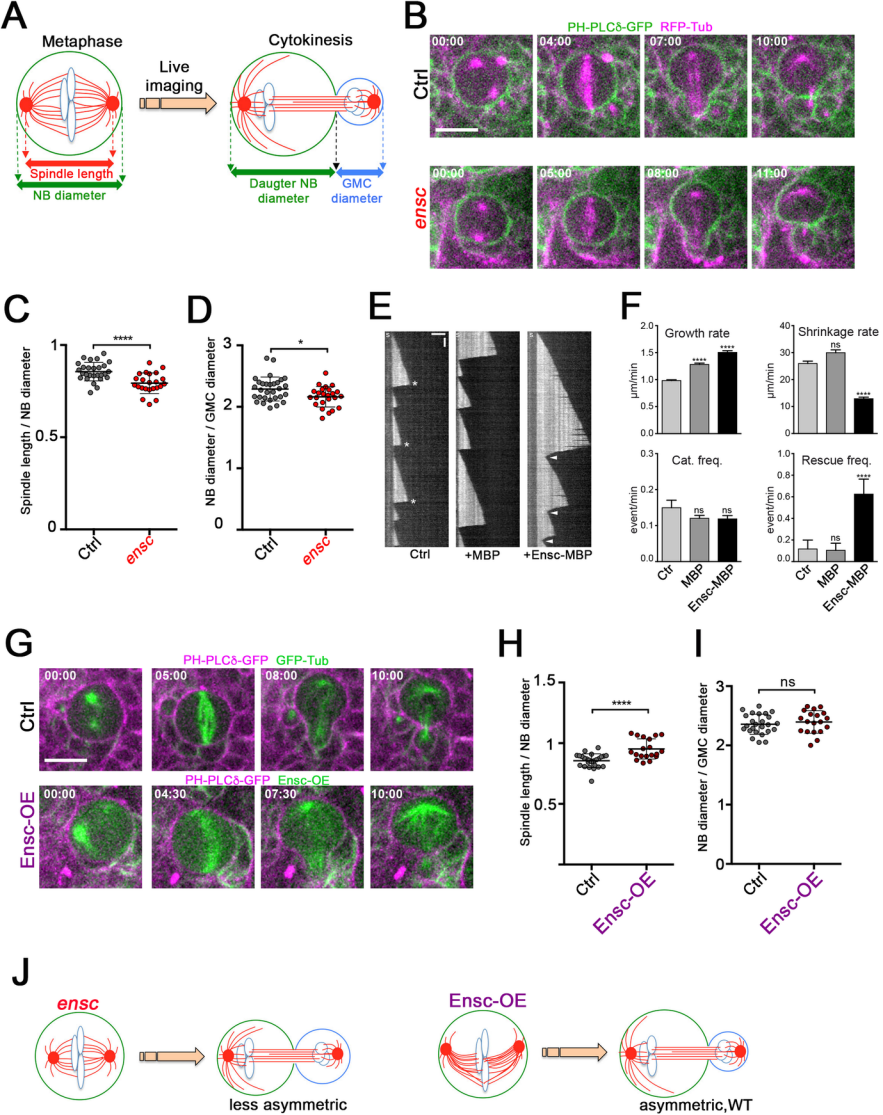


Figure 1

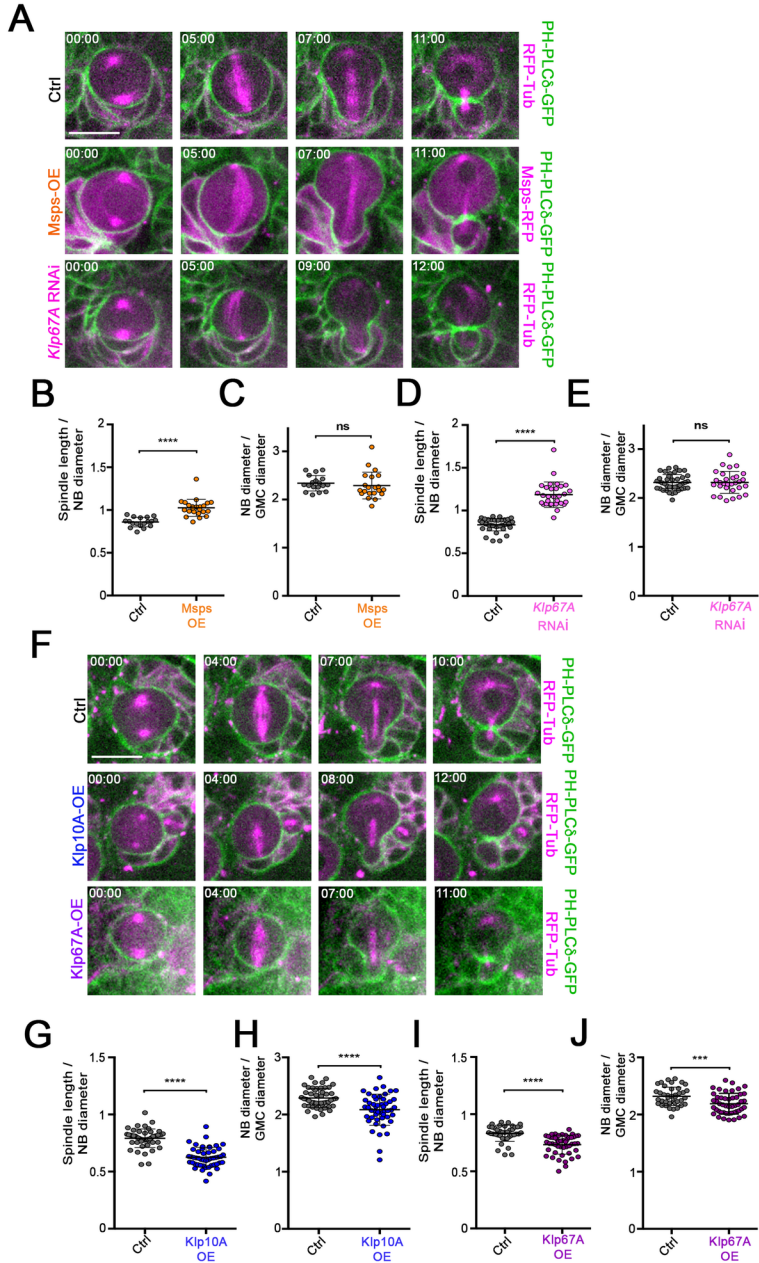


Figure 2

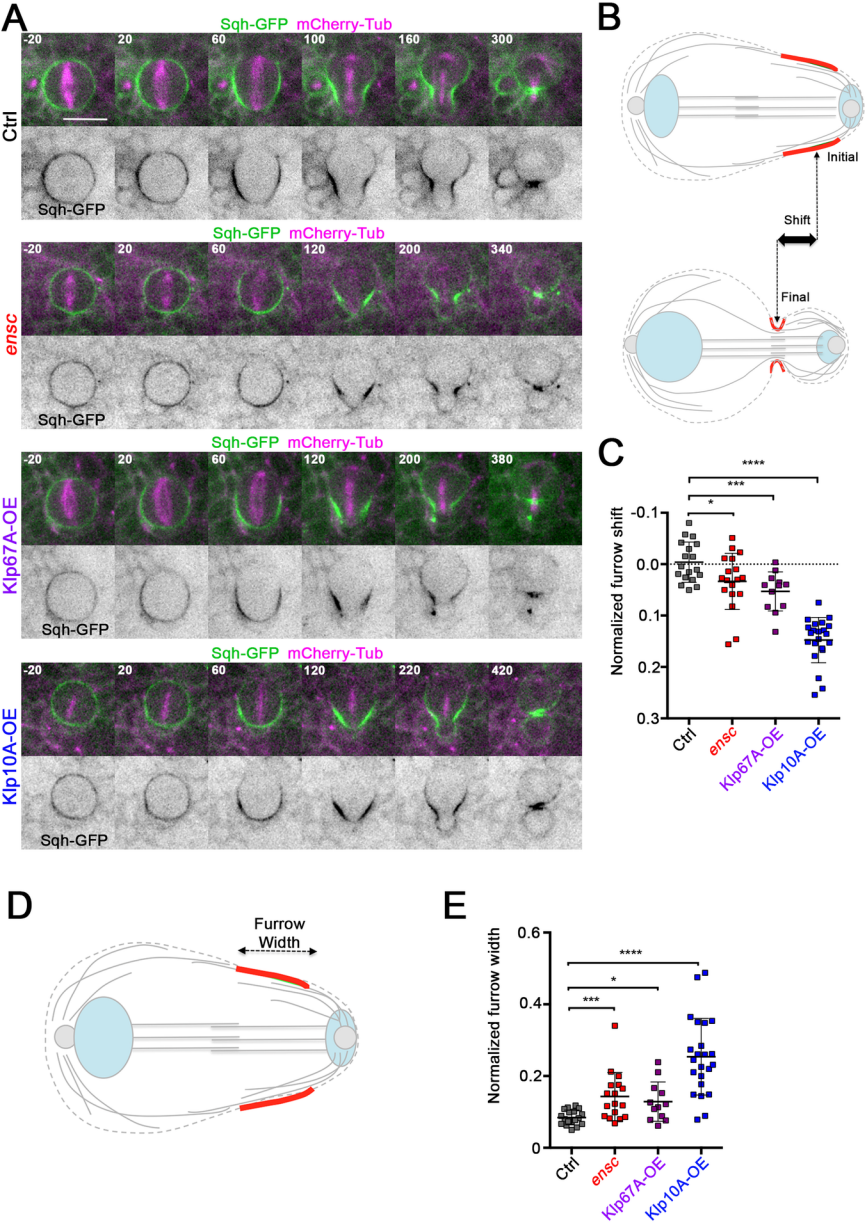


Figure 3

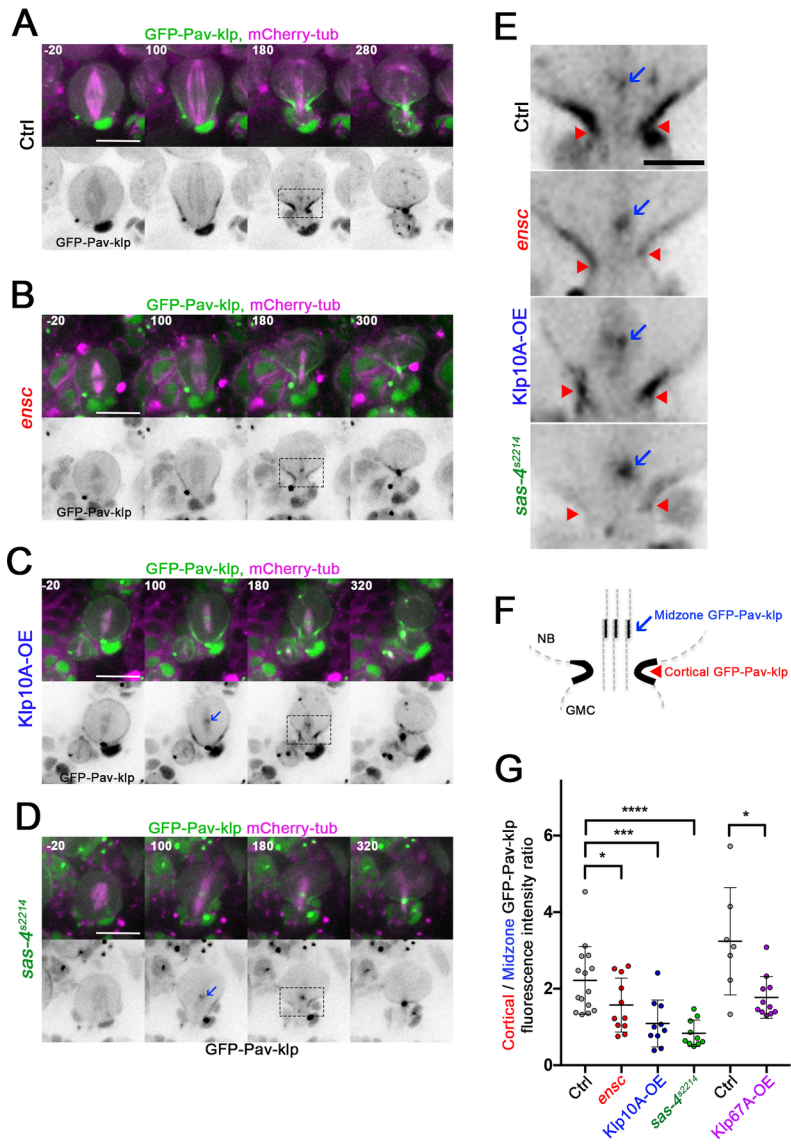


Figure 5

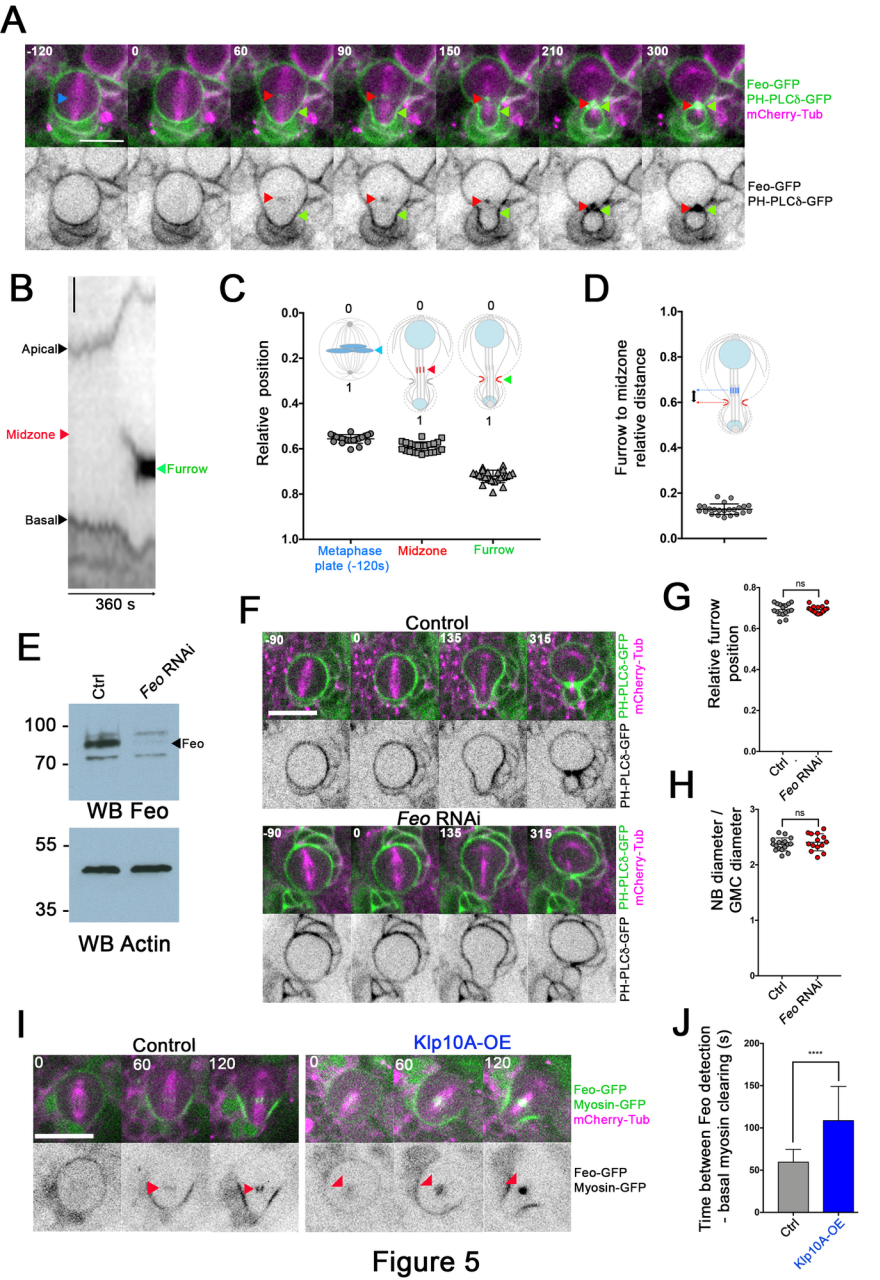
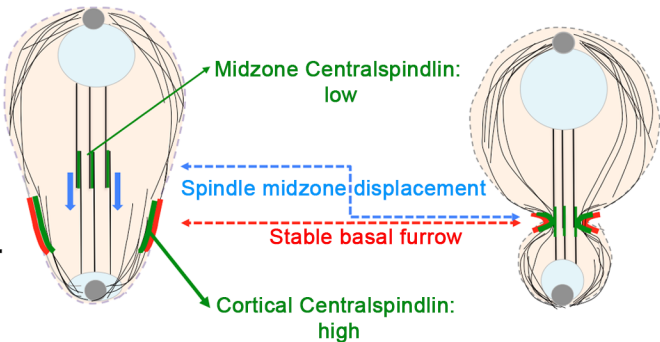


Figure 5

Peripheral astral MTs



Defective astral MTs

