

1 **aPKC-mediated displacement and actomyosin-mediated retention**  
2 **polarize Miranda in *Drosophila* neuroblasts**

3

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9

10 **SUMMARY**

11 Cell fate generation can rely on the unequal distribution of molecules during  
12 progenitor cell division in the nervous system of vertebrates and  
13 invertebrates. Here we address asymmetric fate determinant localization in  
14 the developing *Drosophila* nervous system, focussing on the control of  
15 asymmetric Miranda distribution in larval neuroblasts. We used live imaging of  
16 neuroblast polarity reporters at endogenous levels of expression to address  
17 Miranda localization during the cell cycle. We reveal that the regulation and  
18 dynamics of cortical association of Miranda in interphase and mitosis are  
19 different. In interphase Miranda binds directly to the plasma membrane. At  
20 the onset of mitosis, Miranda is phosphorylated by aPKC and displaced from  
21 the PM. After nuclear envelope breakdown asymmetric localization of Miranda  
22 requires actomyosin activity. Therefore, Miranda phosphorylation by aPKC and  
23 differential binding to the actomyosin network are required at distinct phases  
24 of the cell cycle to polarize fate determinant localization.

## 25 **INTRODUCTION**

26 The development of the central nervous system depends on asymmetric cell  
27 divisions for the balanced production of progenitor as well as differentiating  
28 cells. During vertebrate and invertebrate neurogenesis, cell fates can be  
29 established through the unequal inheritance of cortical domains or fate  
30 determinants during asymmetric progenitor divisions (Knoblich 2008; Doe  
31 2008; Marthiens & ffrench-Constant 2009; Alexandre et al. 2010).

32 An important step in asymmetric cell division is the establishment of a polarity  
33 axis. Asymmetrically dividing *Drosophila* neuroblasts (NBs) establish an axis of  
34 polarity at the onset of mitosis. Like in many other polarized cells, this  
35 depends on the activity of the Par complex (Goldstein & Macara 2007). As  
36 NBs enter prophase, the Par complex including Par3/Bazooka (Baz), aPKC and  
37 Par-6 assembles at the apical NB pole, which drives the localization of fate  
38 determinants to the opposite NB pole establishing the apico basal polarity axis  
39 (Wodarz et al. 1999; Wodarz et al. 2000; Rolls et al. 2003; Petronczki &  
40 Knoblich 2001; Betschinger et al. 2003; Homem & Knoblich 2012; Prehoda  
41 2009).

42 Upon NB division, the basally localized fate determinants segregate to the  
43 daughter cell that commits to differentiation. For this to happen correctly, two  
44 adapter proteins, Partner of Numb (Pon, Lu et al. 1998) and Miranda (Mira,  
45 Ikeshima-Kataoka et al. 1997; Shen et al. 1997), are required. While Pon  
46 localizes the Notch signaling regulator Numb (Uemura et al. 1989; Lu et al.  
47 1998), Mira localizes the homeobox transcription factor Prospero (Pros) and

48 the translational repressor Brat to the basal NB cortex in mitosis (Ikeshima-  
49 Kataoka et al. 1997; Betschinger et al. 2006; C.-Y. Lee et al. 2006). In the  
50 absence of Mira, fate determination is impaired and tumor-like growth can  
51 occur in larval NB lineages (Ikeshima-Kataoka et al. 1997; Caussinus &  
52 Gonzalez 2005).

53 How polarized fate determinant localization in mitotic NBs is achieved is a  
54 long-standing question and despite being a well-studied process, this  
55 mechanism is not fully understood. In embryonic NBs, Mira localization  
56 requires actin (Shen et al. 1998) and myosin activity since mutation in the  
57 myosin regulatory light chain *spaghetti squash (sqh)* (Barros et al. 2003) or  
58 the Myosin VI gene *jaguar* (Petritsch et al. 2003) to lead to Mira localization  
59 defects. Furthermore, in embryos injected with the Rho kinase (ROCK)  
60 inhibitor Y-27632, Mira does not achieve a polarized distribution, which was  
61 rescued by the expression of a phospho-mimetic *sqh* allele. This led to the  
62 proposal that Myosin II plays a critical role in Mira localization and aPKC  
63 affects Mira localization indirectly through regulating Myosin II (Barros et al.  
64 2003).

65 However, it was later shown that Y-27632 can inhibit aPKC directly and that  
66 Mira is a substrate of aPKC (Atwood & Prehoda 2009; Wirtz-Peitz et al. 2008).  
67 In fact many aPKC substrates including Numb and Mira contain a basic and  
68 hydrophobic (BH) motif that can be phosphorylated by aPKC. Upon  
69 phosphorylation the substrates are no longer able to directly bind  
70 phospholipids of the plasma membrane (PM, Bailey & Prehoda 2015; Smith et

71 al. 2007; Dong et al. 2015). Asymmetric Mira localization in mitotic NBs can in  
72 principle be explained by keeping the activity of aPKC restricted to the apical  
73 pole (Atwood & Prehoda 2009). However, is less clear what the contribution  
74 of the actomyosin network to polarized Mira localization is.

75 Intriguingly, Mira localizes uniformly to the cortex of larval brain NBs in  
76 interphase (Sousa-Nunes et al. 2009) before becoming basally restricted in  
77 mitosis. The function and regulation of interphase Mira are unknown. In  
78 embryonic NBs in interphase, Mira and its cargo Pros localize to the apical  
79 cortex (Spana & Doe 1995) and Pros is found in the nucleus of interphase  
80 *mira* mutant NBs (Matsuzaki et al. 1998). Given that the levels of nuclear Pros  
81 in NBs are important for the regulation of entry and exit from quiescence and  
82 NB differentiation (Lai & Doe 2014), interphase Mira localization might be  
83 important in this context.

84 To reveal the regulation of interphase Mira, we set out to determine  
85 differences and similarities in the parameters of Mira binding in interphase  
86 and mitosis and the transition between the two different localizations. Using  
87 fluorescent reporters of the Par complex and Mira at endogenous levels of  
88 expression, we reveal that cortical Mira localization is differently controlled in  
89 interphase, where it uniformly binds to the PM and after nuclear envelope  
90 breakdown (NEB), when actomyosin activity becomes important.

91

92



## 93 **RESULTS**

### 94 **Uniform Miranda is cleared from the cortex during prophase and** 95 **Miranda reappears asymmetrically after NEB**

96 We first confirmed in larval NBs that Mira localizes uniformly to the cortex in  
97 interphase (**Figure 1A**) and co-localizes with its cargo Pros in interphase and  
98 mitosis (**Figure 1 supplement 1**). We further stained *mira* mutant NBs for  
99 Mira and Pros. In these cells, Pros is found in the nucleus in interphase  
100 (**Figure 1 supplement 1**). These results are consistent with a role of Mira in  
101 regulation Pros localization not only in mitosis (Ikeshima-Kataoka et al. 1997),  
102 but also potentially during interphase. We therefore sought to address the  
103 regulation of cortical Mira in interphase and mitosis and the transition  
104 between these localizations.

105

106 To accurately monitor *in vivo* the dynamics of this transition, we used a BAC  
107 construct in which Mira was tagged at its C-terminus with mCherry (Ramat et  
108 al. 2017; **Figure 1 supplement 2**). This tagged Mira recapitulated uniform  
109 cortical localization in interphase (**Figure 1B**, -33 to NEB) and polarized  
110 localization to the basal pole in mitosis (**Figure 1B**, +4), with a 2.5-fold  
111 increase in intensity (n=5, **Figure 1B'**). This transition occurred in two  
112 distinct steps. Firstly, during prophase, after Mira was rapidly excluded from  
113 the apical pole where Baz (**Movie S1**) or aPKC (**Movie S2**) started localizing  
114 (**Figure 1B** -8 to NEB, **B'** -15). Thereafter, Mira was progressively cleared  
115 from most of the rest of the cortex in an apical-to-basal direction (**Figure 1B**,  
116 **B'** -4, -1, respectively to NEB). Secondly, following NEB, Mira reappeared at

117 the cortex in a basal crescent after NEB (**Figure 1B ,B'** +4, +5 respectively).  
118 We further recapitulated these steps using overexpression of GFP::Mira and  
119 by antibody staining of endogenous, non-tagged Mira. The Mira antibody  
120 revealed colcemid insensitive cortical localization in interphase, which was lost  
121 in *mira* mutant NBs (**Figure 1 supplement 3**). Thus, endogenous Mira also  
122 localizes to the cortex independently of microtubules. In conclusion, Mira  
123 transitions from a uniform to a polarized localization by first being cleared  
124 from most of the cortex during prophase, and only upon NEB reappearing in a  
125 basal crescent (**Figure 1C**).

126

## 127 **Actomyosin is required for the establishment and maintenance of** 128 **Miranda crescents after NEB**

129 In embryonic NBs, Mira localization is sensitive to F-actin disruption in mitosis  
130 (Shen et al. 1998). We reasoned that this could be due to defective Mira  
131 clearance in prophase and/or defective Mira reappearance at the basal cortex  
132 after NEB. We tested this by disrupting the actin network of larval NBs with  
133 Latrunculin A (LatA). Despite efficiently disrupting F-actin (**Figure 2**  
134 **supplement**) and causing cytokinesis failure (**Figure 2A**, 3:02, related to  
135 **Movie S3**), LatA treatment affected neither uniform cortical localization of  
136 Mira in interphase (**Figure 2A**, 2:06) nor its clearance during prophase  
137 (**Figure 2A**, 2:21). In contrast, Mira failed to relocalize to a basal crescent  
138 following NEB in LatA-treated NBs (**Figure 2A**, 2:33). We next tested  
139 whether, in addition to crescent establishment, F-Actin controlled crescents  
140 maintenance. To this end, we arrested NBs with colcemid in metaphase (at

141 which point Mira crescents are established), after which we treated them with  
142 LatA. Again, LatA caused Mira to relocalize to the cytoplasm (**Figure 2A**).  
143 Importantly, this relocalization is unlikely to be due to Mira being “swept off”  
144 the cortex by cortical aPKC, which redistributes from an apical crescent to the  
145 entire cortex following LatA treatment (**Figure 2 supplement**). This is  
146 evident since unlike the gradual apical-to-basal clearance of Mira during  
147 prophase (**Figure 1B**), basal Mira crescents fall off the cortex homogenously  
148 upon LatA treatment (**Figure 2 supplement**). Furthermore, Mira falls off the  
149 cortex  $2.8 \pm 1 \text{ min}$  ( $n=13$ ) before aPKC (**Movie S4**) or Baz become detectable  
150 at the basal cortex (**Figure 2 supplement**). Therefore, an intact actin  
151 network is required to establish and maintain asymmetric Mira localization in  
152 mitotic larval NBs.

153

154 Myosin activity has been proposed to be required for Mira localization  
155 (Petritsch et al. 2003; Barros et al. 2003). We tested next which step of Mira  
156 localization involved Myosin. Myosin motor activity relies on the  
157 phosphoregulation of myosin regulatory light chain, encoded by the *sqh* gene  
158 in *Drosophila* (Jordan & Karess 1997), which we disrupted by applying ML-7,  
159 a specific inhibitor of the myosin light chain kinase (MLCK, Bain et al. 2003).  
160 As with LatA, the ML-7 treatment in cycling neuroblasts affected neither  
161 uniform cortical localization of Mira in interphase (**Figure 2B**, 0') nor its  
162 clearance during prophase (**Figure 2B**, 138-278') but resulted in failure to  
163 establish a basal crescent after NEB, which was restored upon drug washout  
164 (**Figure 2B**, 328'). In colcemid-arrested NBs, the ML-7 treatment also

165 resulted in Mira redistributing from the basal crescent to the cytoplasm, which  
166 was restored upon ML-7 washout. Furthermore, unlike LatA treatment, ML-7  
167 did not cause the Par complex to redistribute to the entire cortex (**Figure 2C**,  
168 **Movie S5**). Finally, as in (Das & Storey 2014), we demonstrated the  
169 specificity of the effect of ML-7 by counteracting its effect with a  
170 phosphomimetic version of Myosin regulatory light chain, Sqh. Overexpressing  
171 a phosphomimetic version of Sqh (Sqh<sup>EE</sup>, (Winter et al. 2001) significantly  
172 delayed loss of cortical Mira after ML-7 addition in colcemid arrested NBs  
173 (**Figure 2D**, **Movie S6**).

174 In conclusion, the contribution of F-Actin (Shen et al. 1998) and Myosin  
175 (Petritsch et al. 2003; Barros et al. 2003) to asymmetric Mira localization is  
176 linked to the relocalization of Mira and its maintenance at the basal cortex  
177 following NEB, but not to the control of uniform cortical localization in  
178 interphase or to clearance of Mira during prophase.

179

180 **Defective clearance of interphase cortical Miranda by aPKC results in**  
181 **the persistence of uniform Miranda in mitosis.**

182 NBs mutant for aPKC also fail to asymmetrically localize Mira in mitosis (Rolls  
183 et al. 2003), prompting us to investigate which aspect of Mira localization  
184 involved aPKC in larval NBs. Like in controls, Mira localized uniformly to the  
185 cortex of *apkc*<sup>k06403</sup> (Wodarz et al. 2000) mutant NBs in interphase. However,  
186 it did not clear from the cortex during prophase and instead remained  
187 uniformly localized throughout mitosis (**Movie S7**, **Figure 3A**). Additionally,  
188 expressing a constitutively active form of aPKC, aPKC<sup>AN</sup>, resulted in the loss of

189 uniform cortical Mira binding in interphase (**Figure 3 supplement**). These  
190 results show that during prophase, aPKC negatively regulates the uniform  
191 cortical localization of Mira observed in interphase. They also suggest that the  
192 failure to asymmetrically localize Mira in mitosis in aPKC mutants is due to  
193 defective clearance in prophase resulting in the abnormal persistence of  
194 uniform cortical Mira throughout mitosis.

195

196 We reasoned that, if this were the case, Mira localization in interphase should  
197 have the same characteristics as Mira localization in metaphase upon loss of  
198 function of aPKC. Consistent with this view, unlike Mira localization in mitosis,  
199 but similar to Mira localization in interphase (**Figure 2A**), Mira localization to  
200 the cortex in mitosis was insensitive to LatA treatment in NBs depleted for  
201 aPKC (**Figure 3 supplement**). To corroborate these results we used  
202 fluorescence redistribution after photo-bleaching (FRAP) to analyze the lateral  
203 diffusion/cytoplasmic exchange dynamics of Mira (**Figure 3B**).

204

205 In controls, Mira redistributed about three times faster in interphase  
206 compared to mitosis (**Figure 3C, C'**), suggesting that Mira dynamics are  
207 indeed a relevant characteristic to analyze cell cycle-dependent control of  
208 Mira localization. Yet, in apparent contradiction with our proposition, while  
209 Mira redistribution became faster when aPKC was knocked down by RNAi (or  
210 Lgl<sup>3A</sup> overexpression, known to inhibit aPKC, Betschinger et al. 2003), these  
211 conditions did not result in Mira redistribution in mitosis becoming as fast as  
212 in interphase (**Figure 3C, C'**). However, changes in the actin network caused

213 by progression through the cell cycle (Ramanathan et al. 2015) can influence  
214 dynamics of membrane-associated proteins in general (Heinemann et al.  
215 2013). This is also the case in NBs, as a photo-convertible membrane-  
216 associated reporter that attaches to the entire NB PM via a myristoylation  
217 signal (myr-Eos) showed similar slowing of dynamics in mitosis compared to  
218 interphase (~four-fold, **Figure 3D, D'**).

219

220 Thus, this general cell cycle-driven change of dynamics may account for the  
221 difference between Mira redistribution in interphase and in mitosis in aPKC-  
222 impaired NBs. We tested this by cancelling this general change by treating  
223 mitotic NBs with LatA, which resulted as expected in myr-Eos dynamics falling  
224 into ranges similar to interphase (**Figure 3D, D'**). In aPKC-impaired NBs, the  
225 same LatA treatment resulted in Mira redistribution rates becoming as fast as  
226 in interphase (Figure 3C, C'), suggesting that aPKC loss of function indeed  
227 leads to the persistence of the same dynamics as in interphase (**Figure 3C,**  
228 **C'**). Importantly, myr-Eos dynamics were not sensitive to Lgl<sup>3A</sup> overexpression  
229 alone, arguing against the possibility that aPKC impairment causes general  
230 changes in the cortex that could explain accelerated redistribution of Mira in  
231 this condition.

232 In conclusion, instead of being cleared, Mira persists throughout mitosis with  
233 the same actin-insensitive uniform localization and the same dynamics as in  
234 interphase in *apkc* mutant NBs.

235

236

237 **Miranda binds uniformly to the plasma membrane in interphase**

238 In *Drosophila* S2 cells Mira binds directly to phospholipids of the PM via its BH  
239 motif and the ability to bind the PM is abolished upon phosphorylation of this  
240 motif by aPKC (Bailey & Prehoda, 2015). Our results, that Mira cortical  
241 localization in interphase is actin-independent and that its clearance in  
242 prophase is aPKC dependent, suggest that in interphase Mira is retained  
243 uniformly at the PM by its BH motif.

244

245 Five aPKC phosphorylation sites have been identified in Mira, only one of  
246 which Serine 96 (S96) directly resides in the BH motif. To investigate its  
247 influence on dynamic Mira localization in neuroblasts, we used CrispR to  
248 generate mCherry-tagged versions of Mira control (S96, ctrl, able to rescue  
249 embryonic lethality; see **Figure 1 supplement 2**), a phosphomutant version  
250 (S96A, homozygous embryonic lethal), a phosphomimetic version (S96D,  
251 homozygous embryonic lethal) - shown *in vitro* to reduce phospholipid  
252 binding and Mira recruitment to the PM when overexpressed in S2 cells  
253 (Bailey & Prehoda 2015) - and a complete deletion of the BH motif ( $\Delta$ BH,  
254 homozygous embryonic lethal).

255

256 The control, S96, localized uniformly to the cortex in interphase, was cleared  
257 from the cortex in prophase and reappeared as a basal crescent after NEB  
258 (**Figure 4A, Movie S8**). In contrast, while S96A localized uniformly to the  
259 cortex in interphase, it was not cleared at the onset of prophase when it  
260 presented a transient apical enrichment, perhaps explainable by abnormally

261 stable interactions with apically localized aPKC. After NEB, S96A, localized  
262 however uniformly to the cortex (**Figure 4A, Movie S9**), which occurred  
263 independently of F-actin (**Figure 4B**). Furthermore, S96D did not localize to  
264 the cortex in interphase and instead accumulated predominantly on cortical  
265 microtubules, as evidenced by its relocalization to the cytoplasm upon  
266 colcemid treatment (**Figure 4B**). Nonetheless, S96D always localized  
267 asymmetrically at the cortex following NEB, despite at reduced levels (**Figure**  
268 **4A, Movie S10**).

269

270 Finally, deletion of the BH motif abolished both uniform cortical localization in  
271 interphase and asymmetric cortical localization after NEB (**Figure 4A, Movie**  
272 **S11**, see **Figure 4C** for quantification and **Figure 4D** for summary of the  
273 localization of the different Mira mutants in interphase and mitosis).  
274 Therefore, our findings are consistent with Mira being bound via its BH motif  
275 to the phospholipids of the PM in interphase, and that phosphorylation of S96  
276 by aPKC in prophase disrupts this interaction.

277

278 **Mira crescent size is affected by a Y-27632-sensitive mechanism**  
279 **that operates before NEB**

280 So far, our results demonstrate that in interphase Mira binds uniformly the  
281 PM, from which it is cleared by aPKC at the onset of prophase. However,  
282 upon NEB Mira localization requires actomyosin. This raises the question  
283 whether aPKC contributes to Mira asymmetric localization after NEB.  
284 Temporally controlled inactivation can be achieved with temperature sensitive



285 (ts) alleles or small molecule inhibition. We find that the available ts allele of  
286 aPKC (Guilgur et al. 2012) is hypomorphic already at permissive temperature  
287 resulting in Mira localization defects (not shown). Therefore, we made use of  
288 the non-specific effects of the ROCK inhibitor Y-27632, which inhibits aPKC  
289 with an  $IC_{50}$  of  $10\mu\text{M}$  (Atwood & Prehoda 2009). When we added  $50\mu\text{M}$  of Y-  
290 27632 to already polarized NBs, after 1h in the presence of the drug, we did  
291 not detect any significant changes in Mira or aPKC crescent size ( $n=22$ ,  
292 **Figure 5A**, and **Figure 5 supplement**).

293

294 In striking contrast, when we added half that dose to cycling NBs, while aPKC  
295 crescent size was comparable to controls (**Fig 5 supplement**) Mira crescents  
296 formed, but were significantly enlarged. As a consequence daughter cell size  
297 was also increased ( $n=12$ , **Figure 5B**, **B'**). Intriguingly, such enlarged  
298 crescents were LatA and ML-7 sensitive (**Figure 5C**, see **Figure 5D** for Mira  
299 crescent size quantification), both indicators that enlarged Mira crescents  
300 were not due to lack of phosphorylation of Mira by aPKC. However, when we  
301 titrated the Y-27632 concentration to induce uniform cortical Mira when  
302 added to cycling NBs, Mira became uniformly cortical and was insensitive to  
303 LatA treatment in the presence of  $200\mu\text{M}$  Y-27632, suggesting that this  
304 concentration can lead to inhibition of Mira phosphorylation by aPKC (**Movie**  
305 **S12**,  $n=25$  and not shown).

306

307

308 We then treated colcemid arrested NBs with 200 $\mu$ M Y-27632. It took on  
309 average 52 $\pm$ 11min (n=15) until faint Mira signal became detectable apically.  
310 Nevertheless, Mira distribution remained always biased towards the basal pole  
311 in the presence of Y-27632, an asymmetry that was lost only when we  
312 additionally added LatA, which caused Mira to become uniformly distributed  
313 on the PM (**Figure 5E**, **Movie 13**, n=15). Therefore, 200 $\mu$ M Y-27632 when  
314 added to colcemid arrested NBs, may have an effect on Mira phosphorylation  
315 by aPKC, causing ectopic accumulation of Mira apically. However, given the  
316 non-specific nature of Y-27632 especially at high concentrations, a role for  
317 aPKC cannot be precisely determined in this way.

318 Nonetheless, when added at lower concentrations (25 $\mu$ M) before NEB, Y-  
319 27632 has an effect on Mira crescent size that is unlikely to be caused by  
320 failure of aPKC to phosphorylate Mira.

321

## 322 **Discussion**

323 The establishment of different cell fates in the developing nervous system can  
324 rely on asymmetric distribution of molecules. In this study, we reinvestigated  
325 *in vivo* the precise contributions of aPKC and actomyosin to the localization of  
326 the cell fate determinant adapter Mira during the cell cycle of *Drosophila*  
327 larval NBs. Using live cell imaging we reveal a stepwise contribution of aPKC  
328 and actomyosin to Mira localization. First, Mira was cleared from most of the  
329 cortex during prophase; second, Mira came back to the cortex as a basal  
330 crescent following NEB (**Figure 1B-C**). We then proceeded to assess the  
331 relative contributions of aPKC and actomyosin to these steps. Our study

332 reveals that the central elements of two models proposed to explain Mira  
333 asymmetry, requiring actomyosin (Barros et al. 2003) or involving direct aPKC  
334 phosphoregulation (Atwood & Prehoda 2009) are both correct. Our time-  
335 resolved analysis further allowed determining when in the cell cycle the  
336 proposed mechanisms operate to establish asymmetric fate determinant  
337 localization.

338

339 During interphase, Mira localization does not require the actin cortex, but the  
340 BH motif and Serine 96 (S96) in particular (**Figure 4A**). Thus, in interphase  
341 NBs Mira binds with its BH motif to phospholipids of the PM (Bailey & Prehoda  
342 2015). At the onset of prophase Mira is driven off the PM into the cytoplasm  
343 by aPKC since mutating S96 to Alanine prevents clearance and F-actin-  
344 independent Mira uniformly binding to the PM persists, which is also true in  
345 mitotic *apkc* NBs (**Figure 3A, Figure 3 supplement**). In contrast,  
346 clearance occurs in LatA or ML-7 treated NBs (**Figure 2A,B**). Therefore, aPKC  
347 rather than actomyosin dependent processes drive Mira clearance.

348

349 In the transition from interphase to mitosis, the NBs cytoskeleton gets  
350 remodeled, slowing the diffusion of PM-bound proteins (**Figure 3D**). This  
351 stage is also sensitive to low doses of the ROCK inhibitor Y-27632 (**Figure**  
352 **5B**) and the dynamics of Mira clearance appear altered when LatA treated,  
353 cycling NBs enter mitosis (**Movie S3**). Thus, as in the *C.elegans* zygote  
354 where actomyosin remodeling contributes to the establishment of polarity

355 (Munro et al. 2004), actomyosin remodeling during prophase might also  
356 contribute in NBs to polarize Mira.

357

358 After NEB Mira asymmetry requires actomyosin activity (**Figure 2A-D**).

359 Barros et al. (2003) suggested that myosin II is required to exclude Mira from

360 the apical pole pushing Mira it into daughter cells during division. Our results

361 rather suggest that, myosin activity keeps Mira anchored basally (**Figure 2C**).

362 The observation that NEB is a critical time point for basal crescent formation

363 is further in line with the proposition that factors released from the nucleus

364 upon NEB could regulate Mira localization (Zhang et al. 2016). Interestingly,

365 ROCK and MLCK both affect myosin activity (Amano et al. 1996; Ueda et al.

366 2002; Saitoh et al. 1987; Watanabe et al. 2007) yet the effects of MLCK (ML-

367 7) and ROCK (Y-27632) inhibition on Mira differ (**Figure 2** versus **Figure 5**).

368 In MCDK II cells, Y-27632 and ML-7 treatment has different effects on myosin

369 regulatory light chain phosphorylation (Watanabe et al. 2007). This could

370 result in different effects on myosin activity that may explain the different

371 effects on Mira also in NBs.

372

373 Another question raised by our results is whether aPKC contributes to Mira

374 localization following NEB. We attempted to test this by using the ROCK

375 inhibitor Y-27632, also shown to inhibit aPKC (Atwood & Prehoda 2009). High

376 doses (200 $\mu$ M) of Y-27632 applied to cycling NBs recapitulated defective

377 clearance of Mira by aPKC (**Movie S12**), and the same dose applied to

378 already polarized NBs resulted in a partial loss of Mira asymmetry, however

379 after a ~50min delay (**Figure 5E, Movie 13**). Thus, aPKC may be required  
380 after NEB to reinforce Mira asymmetry by excluding it apically, as suggested  
381 (Atwood & Prehoda 2009). Low doses (25 $\mu$ M) of Y-27632, despite not  
382 inhibiting aPKC, affect Mira crescent size in cycling NBs (**Figure 5B**). Thus, Y-  
383 27632 also inhibits other aPKC-independent mechanisms, making unclear  
384 whether the partial loss of Mira asymmetry in already polarized NBs treated  
385 with high Y-27632 is solely due to the disruption of a post-NEB aPKC function.  
386 Addressing this question in the future will require specific and temporally  
387 controlled aPKC inhibition.

388

389 When the BH motif is deleted, Mira cannot form crescents (**Figure 4A**). We  
390 recently identified that maintenance of Mira crescents also requires interaction  
391 of Mira with its cognate mRNA (Ramat et al. 2017). Thus, establishment of  
392 Mira crescents and their maintenance are differently controlled. The BH motif  
393 might be important to initiate Mira asymmetric localization, while actomyosin  
394 dependent processes contribute to establishment and stabilize Mira in a  
395 subsequent maintenance step.

396

397 Finally, a question of interest is why would Mira localization to the cortex be  
398 controlled by different mechanisms in interphase and mitosis. Mira co-  
399 localizes with Pros in interphase (**Figure 1 supplement 1**). It is possible that  
400 Mira sequesters Pros, helping to prevent its nuclear localization. In this way  
401 Pros induced cell cycle exit could be prevented (Choksi et al. 2006; Lai & Doe  
402 2014). Nuclear Pros is developmentally controlled by hedgehog signaling and

403 the temporal transcription factor cascade (Chai et al. 2013; Maurange et al.  
404 2008) and regulated by RanGEF BJ1 (Joy et al. 2014). However, the  
405 underpinning cell biology is not clear. Being able to regulate Miras interaction  
406 with the PM and at the actomyosin cortex differentially might allow  
407 controllable segregation of fate determinants to daughter cells while  
408 permitting tuning of nuclear Pros levels through its interaction with Mira in  
409 interphase.

410

## 411 **Materials & Methods**

### 412 *Fly stocks and genetics*

413 Flies were reared on standard corn meal food at 25 degrees. Lines used:  
414 (1) Baz::GFP trap (Buszczak et al. 2007); (2)  $w^{1118}$  (Bloomington); (3)  
415 MARCM: hsFlp tubGal4 UASnlsGFP; FRT42B tubGal80/Cyo and FRT82B gal80  
416 (T. Lee & Luo 1999); (4) worniu-Gal4 (Albertson et al. 2004); (5) UAS-  
417 Lgl<sup>3A</sup>::GFP (Wirtz-Peitz et al. 2008); (6) UAS-Lgl<sup>3A</sup> (Betschinger et al. 2003);  
418 (7)  $w^{1118}, y, w, hsp70-flp; tubP-FRT > cd2 > FRT-Gal4, UAS-GFP$  (Gift from M.  
419 Gho); (8) Mz1061 (Ito et al. 1995); (9) UAS-GFP::Mira (Mollinari et al. 2002).  
420 (10) FRT82B  $mira^{L44}$  (Matsuzaki et al. 1998). (10) Df(3R)ora<sup>19</sup> (Shen et al.  
421 1997). (11) UAS-aPKC<sup>RNAi</sup>:  $P\{y[+t7.7] v[+t1.8]=TRiP.HMS01320\}attP2$   
422 (BL#34332); (12) Numb::GFP (Couturier et al. 2013); (13) FRT42B  $apkc^{k06403}$   
423 (Wodarz et al. 2000); (14) UAS-aPKC<sup>ΔN</sup>; (15)  $P\{UASp-sqh.E20E21\}3$   
424 (BL#64411); (16)  $P\{10xUAS-IVS-mye::tdEos\}attP2$  (BL #32226);  $y[1] w[*];$   
425  $P\{y[+t*] w[+mC]=UAS-Lifeact-Ruby\}VIE-19A$  (BL# 35545); (17) aPKC::GFP  
426 (Besson et al. 2015); *Source 1 of Mira::mCherry: BAC{mira::mcherry-MS2}*  
427 (Ramat et al. 2017). aPKC<sup>RNAi</sup> clones were generated by heat-shocking larvae  
428 of the genotype  $y, w, hsp70-flp; tubP-FRT > cd2 > FRT-Gal4, UAS-GFP;$   
429  $P\{y[+t7.7] v[+t1.8]=TRiP.HMS01320\}attP2$ . Heat shocks were performed  
430 24hph and 48hph for 1 hour at 37°C. MARCM clones were generated by heat  
431 shocking L1 larvae for 2h at 37°C.

432 Generation of Mira alleles: Source 2 of Mira::mCherry: *mira*<sup>mCherry</sup>;  
433 *mira*<sup>ΔBHmCherry</sup> (Ramat et al. 2017), *mira*<sup>S96A-mCherry</sup> and *mira*<sup>S96D-mCherry</sup> are  
434 derived from *mira*<sup>KO</sup> (Ramat et al. 2017). *mira*<sup>mCherry</sup> was generated by  
435 inserting a modified wt genomic locus in which mCherry was fused to the C-  
436 terminus following a GSAGS linker into *mira*<sup>KO</sup>. For *mira*<sup>S96D-mCherry</sup>: TCG  
437 (Serine96) was changed to GAC (aspartic acid). For *mira*<sup>S96A-mCherry</sup>: TCG was  
438 replaced with GCG (alanine). CH322-11-P04 was the source for the *mira*  
439 sequences cloned using Gibson assembly into the RIV white vector (Baena-  
440 Lopez et al. 2013) that was injected using the attP site in *mira*<sup>KO</sup> as landing  
441 site. *BAC{mira::mcherry-MS2}* (Ramat et al. 2017) **see Figure 1**  
442 **supplement 2**). While *mira*<sup>mCherry</sup> behaves similarly to *BAC{mira::mcherry-*  
443 *MS2}* and rescues embryonic lethality, *mira*<sup>ΔBHmCherry</sup>, *mira*<sup>S96A-mCherry</sup> and  
444 *mira*<sup>S96D-mCherry</sup> are homozygous lethal.

445

446 Live imaging: Live imaging was performed as described (Pampalona et al.  
447 2015). Briefly, brains were dissected in collagenase buffer and incubated in  
448 collagenase for 20 minutes. Brains were transferred to a drop of fibrinogen  
449 (0.2mgml<sup>-1</sup>, Sigma f-3879) dissolved in Schneider's medium (SLS-04-351Q)  
450 on a 25mm Glass bottom dish (WPI). Brains were manually dissociated with  
451 needles before the fibrinogen was clotted by addition of thrombin (100Uml<sup>-1</sup>,  
452 Sigma T7513). Schneider's medium supplemented with FCS, Fly serum and  
453 Insulin was then added. A 3-4μm slice at the center of the neuroblasts was  
454 then imaged every 30-90s using a 100x OIL objective NA1.45 on a spinning  
455 disk confocal microscope. Data was processed and analyzed using FIJI  
456 (Schindelin et al. 2012). For nuclear volume measurements Imaris was used.  
457 All other drugs were added to the media either prior or during imaging: ML-7  
458 (Sigma, I2764, dissolved in water), Y-27632 (Abcam, Ab120129, dissolved in  
459 water). Drugs were washed out by media replacement, in the polarity  
460 reconstitution assay colcemid concentrations were kept constant throughout  
461 the experiments. FRAP experiments were carried out on a Leica SP8 confocal  
462 using a 63x NA1.2 APO water immersion objective. To estimate t<sub>1/2</sub> for the

463 recovery curves we used published curve fitting methods (Rapsomaniki et al.  
464 2012).

465

#### 466 *Immunohistochemistry*

467 *Primary cell culture:* Brains were dissected in collagenase buffer and  
468 incubated for 20 minutes in collagenase, as for live imaging. Brains were then  
469 transferred into supplemented Schneider's medium and manually dissociated  
470 by pipetting up and down. Cells were pipetted onto a poly-lysine coated  
471 25mm glass bottomed dish and left to adhere for 40 minutes. Schneider's was  
472 then replaced with 4% Formaldehyde (Sigma) in PBS and cells were fixed for  
473 10 minutes. Cells were permeabilized with 0.1% PBS-Triton for 10 minutes.  
474 Cells were then washed with PBS 3x 10minutes before antibody staining  
475 overnight at 4°C. All antibodies were dissolved in PBS-1%Tween. *Whole*  
476 *mount brains:* Brains were fixed in 4% Formaldehyde (Sigma) for 20 minutes  
477 at room temperature. Primary antibodies: Rabbit anti-Miranda (1:200, gift  
478 from C. Gonzalez), Mouse anti-GFP (1:400, Abcam). Rabbit anti-Brat (1:200, a  
479 gift from J. Knoblich). Guinea Pig anti-Dpn (1:500 a gift from J. Skeath).  
480 Mouse anti-Pros (1:40, DSHB). To stain F-Actin we used Alexa Fluor 488 or  
481 561 coupled Phalloidin (Molecular Probes, 5:200) for 20 minutes at room  
482 temperature. Secondary antibodies (all from life technologies and raised in  
483 donkey: Anti-Rabbit Alexa-594, Anti-Mouse Alexa-488, Anti-Rabbit Alexa-647,  
484 Anti-Guinea Pig Alexa-647. Microscopy was performed using a Leica-SP8  
485 CLSM (60x Water objective, 1.2) and images were processed using FIJI.

486

487 In all cases the sample size  $n$  provided reflects all samples collected for one  
488 experimental condition. Experimental conditions were repeated at least twice  
489 to account for technical and biological variation.

490

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503

504

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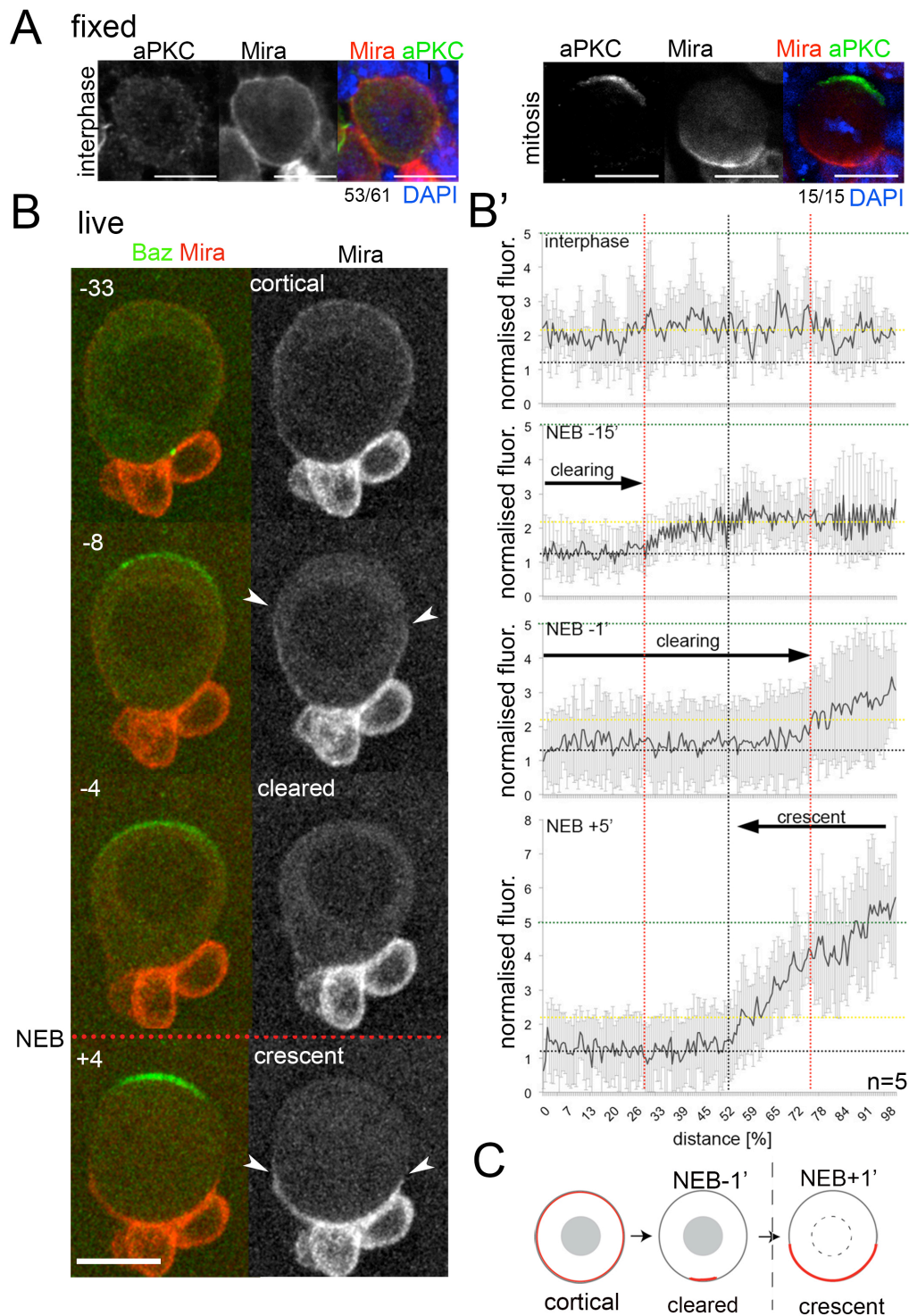
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652 **Figures and Figure legends**

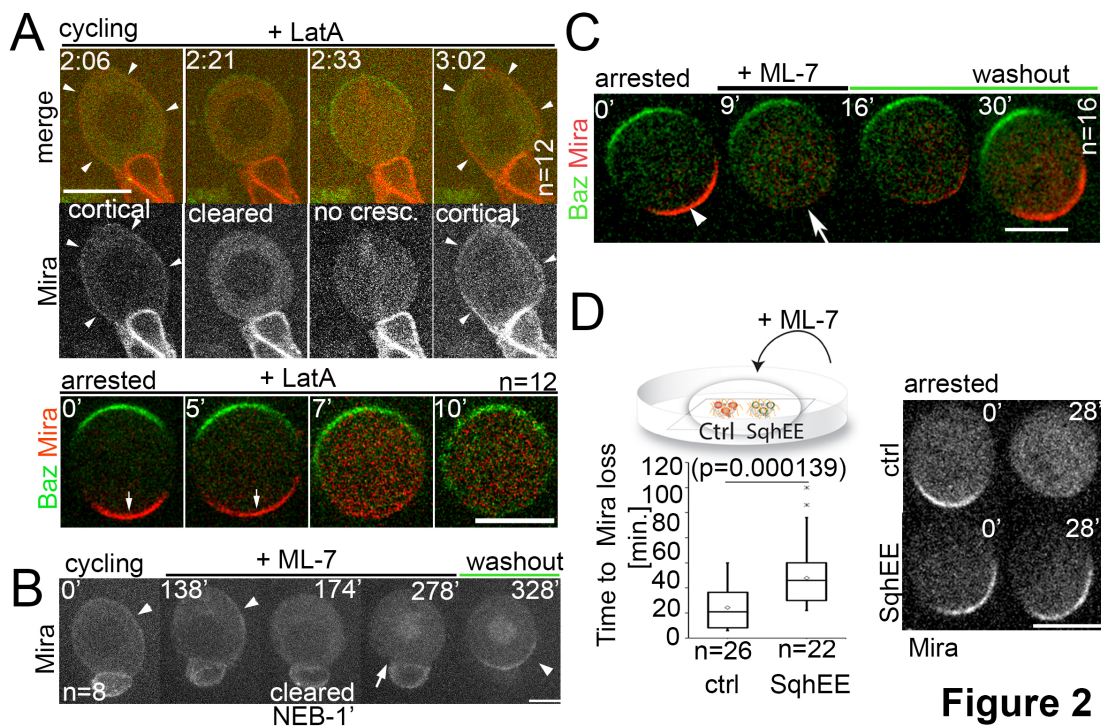


**Figure 1**

653

654 **Figure 1.** Miranda is cleared from the cortex before localizing in a basal  
 655 crescent in mitosis. **(A)** Larval brain NBs fixed and stained as labeled at the  
 656 indicated cell cycle stage. **(B)** Selected frames from **Movie S1**. NB in primary  
 657 cell culture expressing Baz::GFP (green) and Mira::mCherry (red) in the

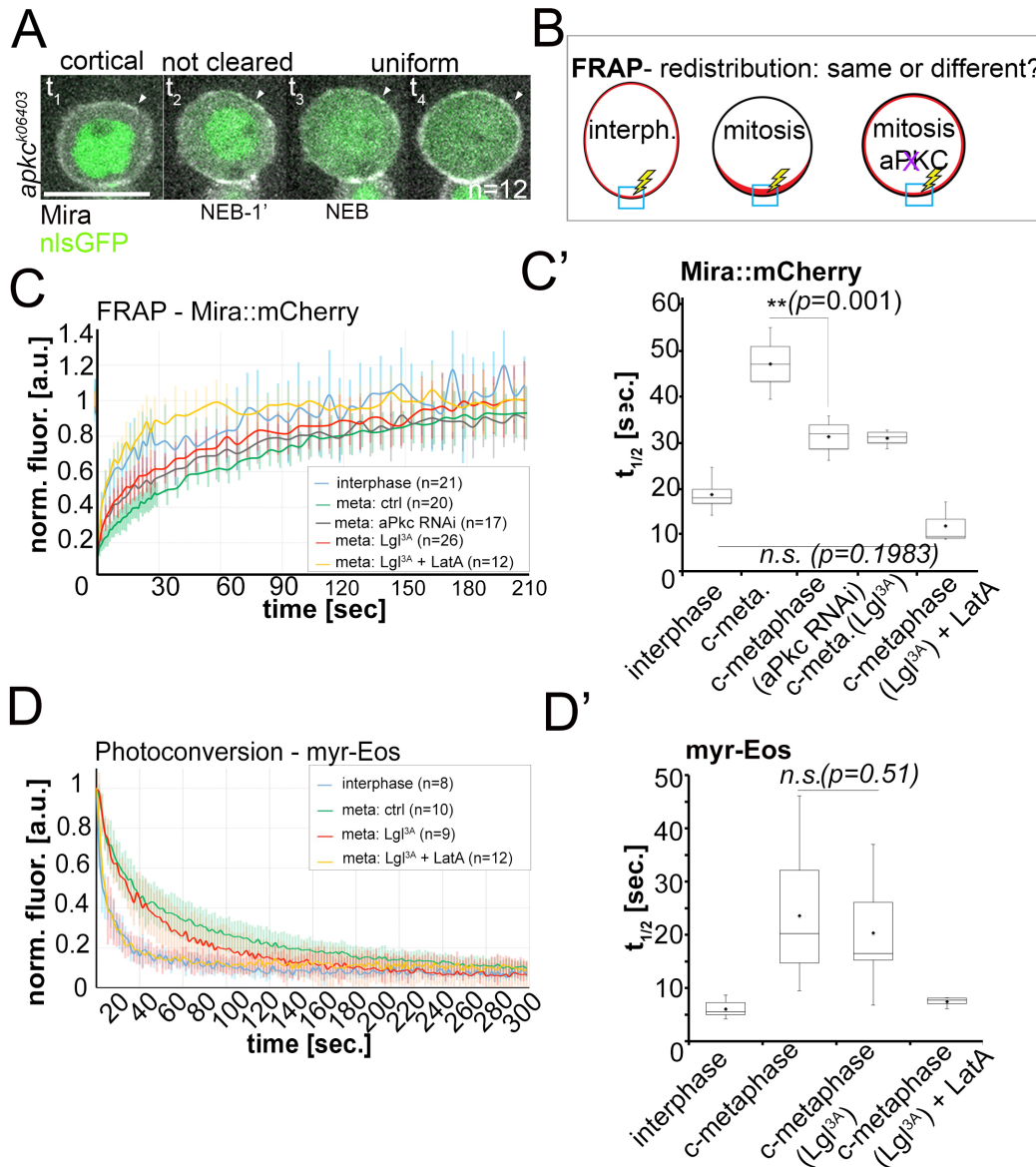
658 transition from interphase to mitosis. Arrowheads point at Mira being cleared  
 659 (-8) and at basal Mira crescent (+4). **(B')** Quantification of cortical  
 660 Mira::mCherry signal plotting the fluorescence intensities from the apical to  
 661 the basal pole computationally straightening (Kocsis et al. 1991) the cortices  
 662 of 5 NBs against the distance in %. Fluorescence was background subtracted  
 663 and normalized to background subtracted cytoplasmic signal (1, dotted line).  
 664 Cortical signal (yellow dotted line) and signal after NEB (green dotted line).  
 665 Error bars, standard deviation. **(C)** Schematic of Mira localization.  
 666 *BAC{mira::mcherry-MS2}* was the source of Mira::mCherry. Scale bar 10 $\mu$ m.  
 667 Time stamp: minutes.  
 668  
 669



**Figure 2**

670  
 671  
 672 **Figure 2.** Differential response of Mira localization in interphase and mitosis  
 673 to disruption of the actin cytoskeleton. **(A)** Stills from **Movie S3**. LatA was  
 674 added to a cycling NB in primary cell culture expressing Baz::GFP (green) and  
 675 Mira::mCherry (red). Arrowheads point at cortical Mira after culturing ~1h  
 676 with LatA (2:06). At 1 min to NEB, Mira::mCherry is cleared from the cortex  
 677 (2:21). Mira forms no crescent in the next mitosis (2:33), but after cytokinesis

678 fails (note bi-nucleated cell at 3:02), Mira is recruited to the cortex  
679 (arrowheads). Bottom panels: Colcemid arrested NBs expressing Baz::GFP  
680 and Mira::mCherry. 5 $\mu$ M LatA was added prior to imaging at 15sec. intervals.  
681 Mira crescents (arrows) are lost upon LatA treatment. **(B)** Cycling NB in  
682 primary cell culture expressing Mira::mCherry, that remains cortical upon ML-  
683 7 addition (15 $\mu$ M; interphase: 0' and 138', arrowheads), is cleared 1minute  
684 prior to NEB (174'), does not form a crescent after NEB (278', arrow), but  
685 accumulates on the spindle (seen in cross section). After ML-7 washout, a  
686 basal Mira::mCherry crescent recovers (arrowhead, 328'). **(C)** Related to  
687 **Movie S5**. Colcemid arrested NB in primary cell culture expressing Baz::GFP  
688 (green) and Mira::mCherry (red). After addition of 20 $\mu$ M ML-7 Mira  
689 (arrowhead, 0') becomes cytoplasmic (arrow, +9'), but upon ML-7 washout a  
690 Mira crescent recovers. **(D)** The effect of 20 $\mu$ M ML-7 can be quenched by  
691 overexpressing a phospho-mimetic form Sqh (Sqh<sup>EE</sup>). Colcemid arrested NBs  
692 (ctrl: Mira::mCherry: Sqh<sup>EE</sup>: Mira::mCherry co-expressing Sqh<sup>EE</sup> by  
693 *worniuGal4*). Ctrl and Sqh<sup>EE</sup> were co-cultured and ML-7 added (related to  
694 **Movie S6**). Quantification of the time required to cause Mira::mCherry to  
695 become cytoplasmic shown on the left. Two-tailed ttest for independent  
696 means revealed significance. *BAC{mira::mcherry-MS2}* was the source of  
697 Mira::mCherry. Scale bar: 10 $\mu$ m.  
698  
699



**Figure 3**

700

701

702

703 **Figure 3.** Lateral diffusion and cytoplasmic exchange of cortical Miranda are

704 different in control and aPKC impaired mitotic NBs. **(A)** Stills from **Movie S7**

705 of an *apkc<sup>k06403</sup>* mutant NB (MARCM clone labeled with nlsGFP, green)

706 expressing Mira::mCherry (grey). Mira is cortical in interphase, as the NB

707 enters mitosis and after NEB (arrowheads,  $t_1 - t_4$ ). **(B)** Conditions analyzed by

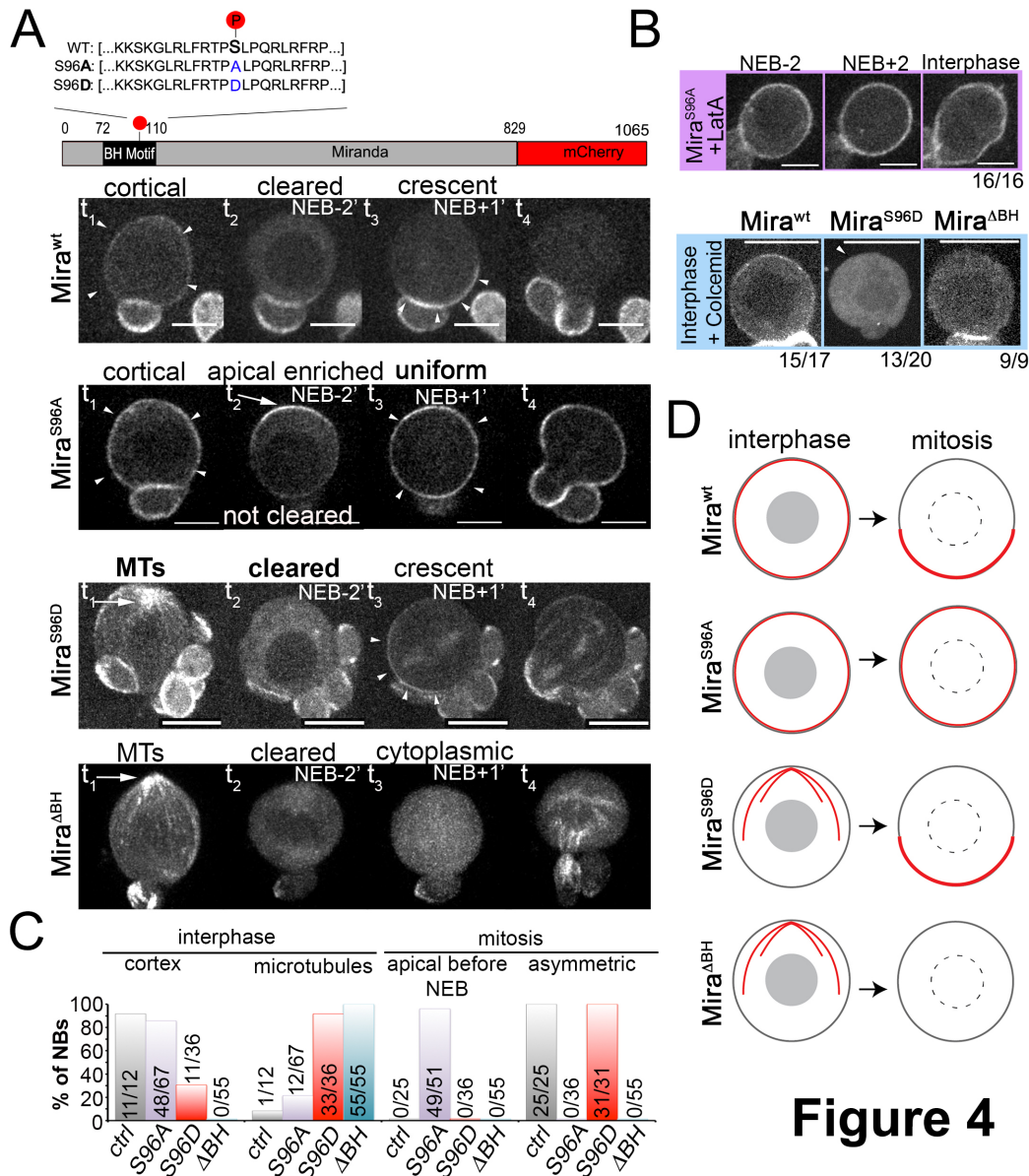
708 FRAP. **(C)** Fluorescence redistribution curves of cortical Mira::mCherry at the

709 indicated conditions. **(C')** Estimates of  $t_{1/2}$  [sec.] for cortical Mira::mCherry

710 under the indicated conditions derived from curve fitting (Rapsomaniki et al.



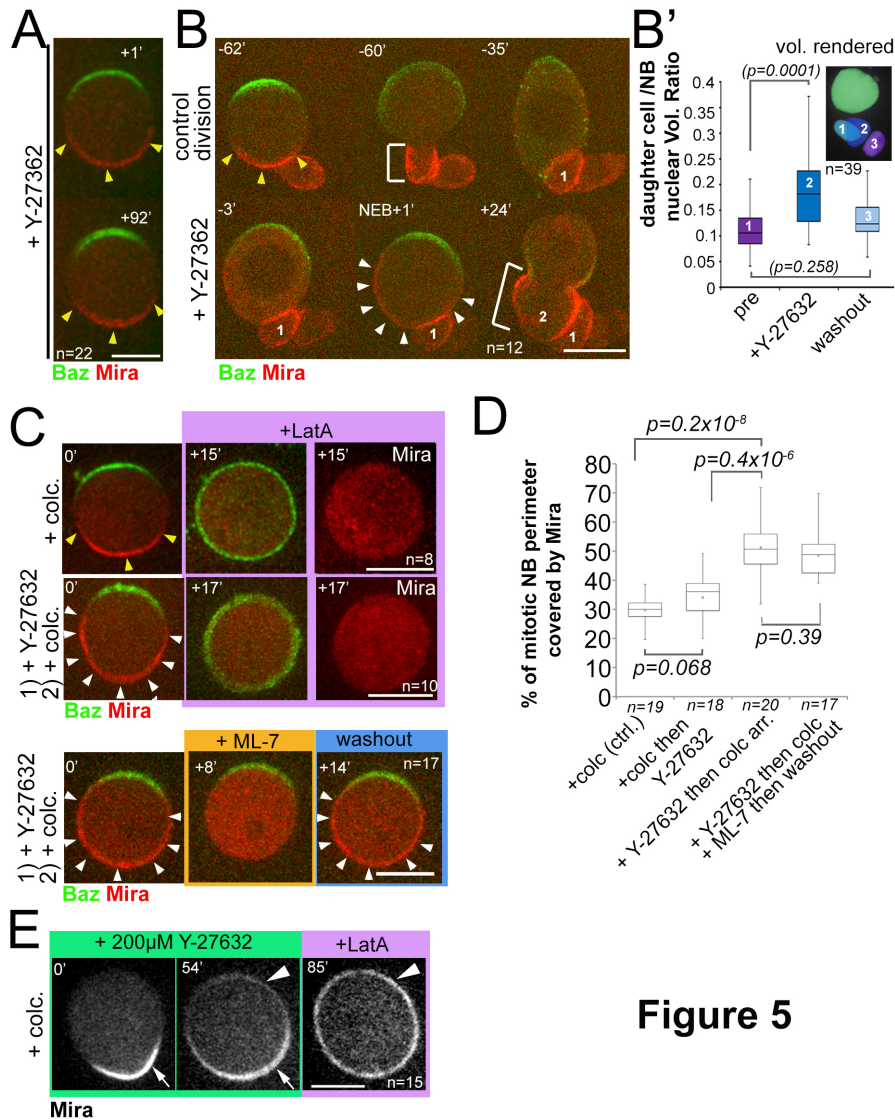
711 2012). (D) Photo-conversion experiment monitoring loss of myr-EOS  
 712 converted signal over time. (D') Estimates of  $t_{1/2}$  [sec.] for cortical  
 713 Mira::mCherry under the indicated conditions from curve fitting.  
 714 Overexpression was driven by *worniu-Gal4*. p values: two-tailed ttest for  
 715 independent means. Scale bar: 10 $\mu$ m.  
 716



**Figure 4**

717  
 718 **Figure 4.** Miranda binds to the plasma membrane in interphase NBs via its  
 719 BH motif. (A) Schematic indicating the different Mira alleles used.  
 720 Mira::mCherry localizes cortically uniform in interphase (arrowheads  $t_1$ ), is  
 721 cleared from the cortex shortly before NEB and forms a crescent (arrowheads  
 722  $t_3$ ) thereafter that is inherited by daughter cells (related to **Movie S8**). The

723 phosphomutant S96A is uniformly cortical in interphase, accumulates apically  
724 shortly before NEB (arrow,  $t_2$ ), is uniformly cortical after NEB (arrowheads  $t_3$ )  
725 and in telophase ( $t_4$ , related to **Movie S9**). The phosphomimetic S96D  
726 localizes to cortical microtubules in interphase (arrow  $t_1$ ), is cleared from the  
727 cortex before NEB and asymmetric after NEB (arrowheads  $t_3$ ) and segregates  
728 to daughter cells (related to **Movie S10**). Deletion of the BH motif leads to  
729 cortical microtubule localization in interphase (arrow  $t_1$ ), cytoplasmic  
730 localization before and after NEB and reappearance on microtubules around  
731 cytokinesis (related to **Movie S11**). **(B)** Neuroblasts expressing the indicated  
732 Mira alleles were treated with  $1\mu\text{M}$  LatA or  $50\mu\text{M}$  colcemid for 60min. Cortical  
733 localization of S96A is insensitive to LatA treatment. Below: While the Control  
734 remains cortical, S96D and  $\Delta\text{BH}$  become cytoplasmic upon colcemid  
735 treatment. **(C)** Frequency of indicated localization of the different Mira  
736 mutants. **(D)** Schematic of the localization of the different Mira alleles. Scale  
737 bar:  $10\mu\text{m}$ .  
738



**Figure 5**

739

740 **Figure 5.** Mira crescent size is affected by a Y-27632-sensitive mechanism  
 741 that operates before NEB. **(A)** Culturing colcemid arrested NBs in 50µM Y-  
 742 27632 did not alter Mira crescent size (yellow arrowheads). **(B)** NBs polarizing  
 743 in the presence of 25µM Y-27632 show enlarged Mira crescents. Control  
 744 division (-62' to -35') with normally sized Mira crescent and daughter cell size  
 745 (-60'; yellow arrowheads, bracket, respectively). Dividing in the presence of  
 746 Y-27632 (-3, NEB+1) leads to an enlarged Mira crescent (NEB+1, white  
 747 arrowheads) and enlarged daughter cell size (+24', brackets, 2). **(B')** Plot of  
 748 the ratio of daughter cell to NB nuclei as a measure for the effect of Y-27632  
 749 on daughter cell size. NBs expressing NLSGFP were imaged by DIC to follow  
 750 daughter cell birth order during three consecutive divisions [1) pre-treatment;  
 751 2) div. in the presence of 25µM Y-27632; 3) div. after drug washout]. Then a

752 high-resolution z-stack of nlsGFP was recorded, and the nuclear volumes  
753 rendered and calculated using IMARIS to plot their ratio. *p* values: Dunn's  
754 test. **(C)** NBs were allowed to polarize in the absence (*upper row*) or presence  
755 of 25 $\mu$ M Y-27632 (middle and lower row) followed by colcemid arrest. *upper*  
756 *row*: Control NB with normal Mira crescent (yellow arrowheads) was  
757 depolarized by 1 $\mu$ M LatA. Mira was displaced into the cytoplasm. *middle row*:  
758 adding 1 $\mu$ M LatA leads to displacement of the enlarged Mira crescent (yellow  
759 arrowheads) in the cytoplasm. *Lower row*: adding 20 $\mu$ M ML-7 drives Mira into  
760 the cytoplasm (+8'). Upon ML-7 washout, Mira recovered to an enlarged  
761 crescent (+14', white arrowheads). **(D)** Quantification of Mira crescent size in  
762 the aforementioned experiments (unpaired ttest). **(E)** Colcemid arrested NBs  
763 were treated with 200 $\mu$ M Y-276322. Mira remains asymmetric even after  
764 56min in the drug. LatA addition (5 $\mu$ M) abolishes that asymmetric bias and  
765 Mira is uniformly distributed on the membrane. *Time* stamp: min. Labels as  
766 indicated. *BAC{mira::mcherry-MS2}* was the source of Mira::mCherry. Scale  
767 bar: 10 $\mu$ m.

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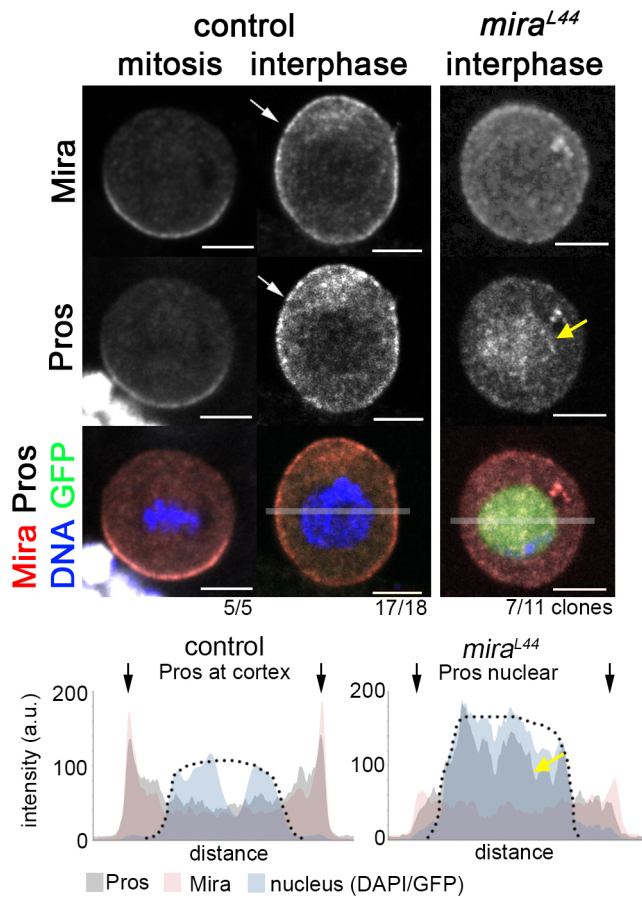
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784 **Figure supplements**

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786

**Figure 1 supplement 1**

787 **Figure 1 supplement 1.** Uniform cortical Prospero depends on Miranda in  
788 interphase larval NBs. In *w*<sup>1118</sup> brains, Mira and Pros form basal crescents in  
789 mitosis and both are cortical in interphase (arrow). In an interphase *mira*<sup>L44</sup>  
790 NB (MARCM clone, GFP<sup>+</sup>) cortical Mira and Pros are strongly reduced and Pros  
791 accumulates in the nucleus (yellow arrow). Transparent bars in merge  
792 interphase (control) and *mira*<sup>L44</sup> indicate area used for plot profiles shown  
793 below. Pros and Mira are at the cortex (arrows) in the control and Pros is  
794 enriched in the nucleus in the mutant (yellow arrow). Arrows: outline of cell.  
795 Dotted line: nucleus (based on DAPI, control and GFP (MARCM clone)). Scale  
796 bar: 10 $\mu$ m.

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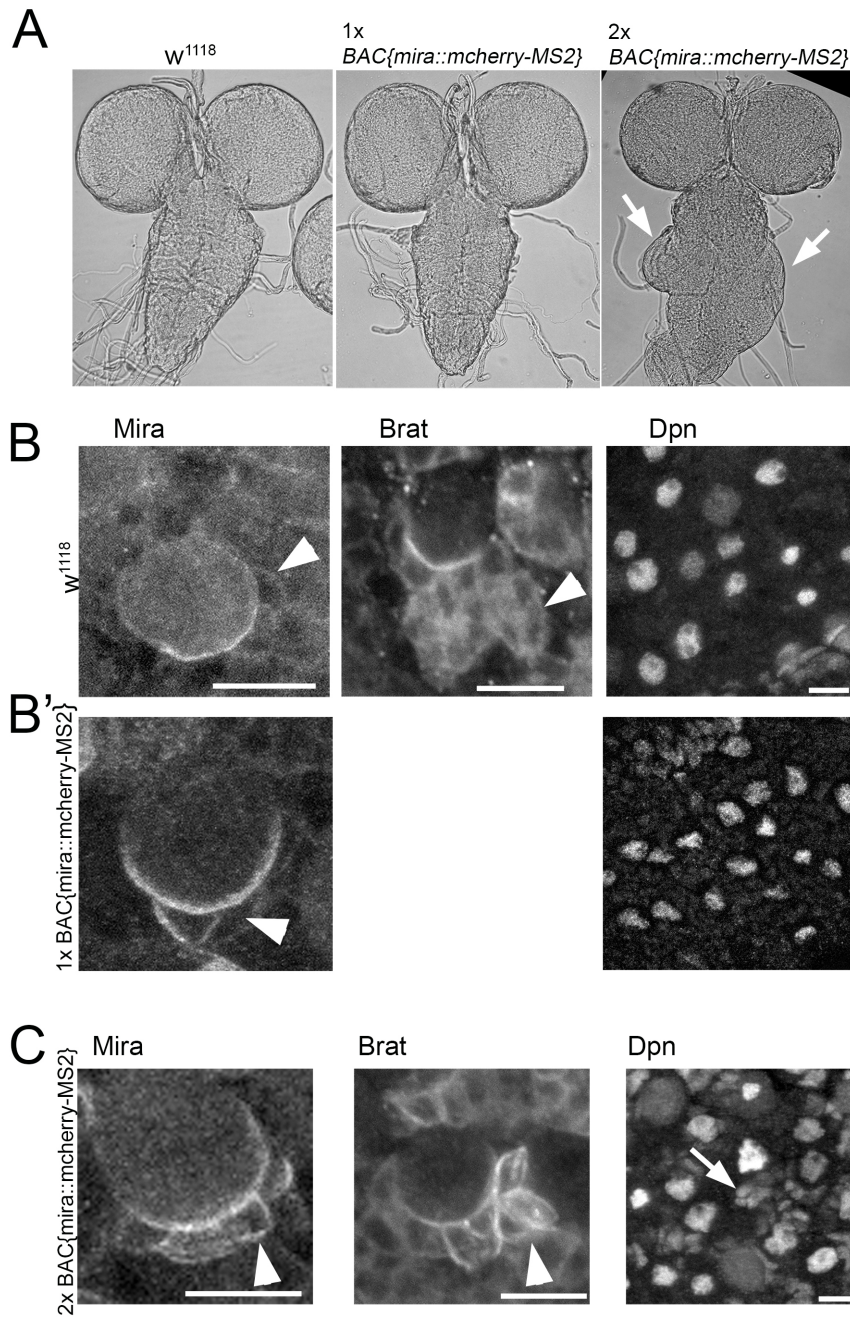


Figure 1 supplement 2

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801 **Figure 1 supplement 2.**  $BAC\{mira::mcherry-MS2\}$  rescues embryonic

802 lethality of the loss of function allele  $mira^{L44}$  over the deficiency  $DF(3R)ora^{19}$ .

803 However, animals die during puparium formation, when  $BAC\{mira::mcherry-$

804  $MS2\}$  is the only source of Mira. **(A)** Brightfield images of fixed whole mount

805 brain preparations.  $w^{1118}$  (control,  $n=5$ ) a  $BAC\{mira::mcherry-MS2\}$  brain

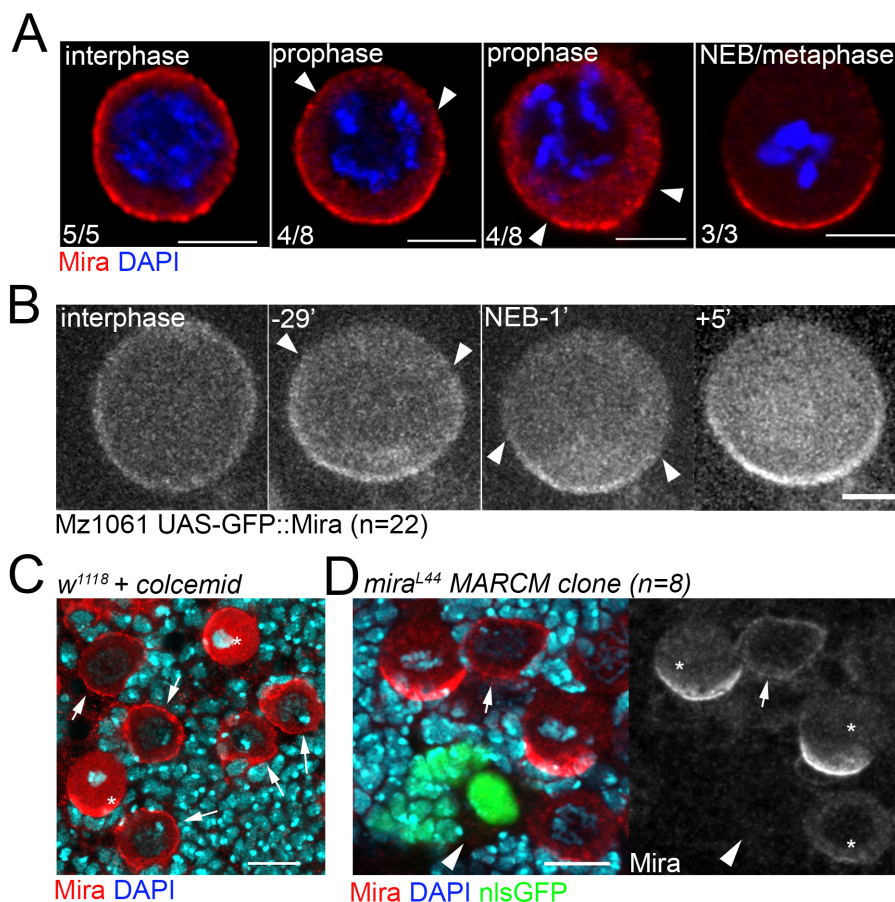
806 over a wild type chromosome (1x  $BAC\{mira::mcherry-MS2\}$ ,  $n=12$ ) and a

807 brain from  $BAC\{mira::mcherry-MS2\}$   $Df(3R)ora^{19}$  over a unrecombined

808  $mira^{BACmCherry}$  chromosome (2x  $BAC\{mira::mcherry-MS2\}$ ,  $n=12$ ). 2x

809 *BAC{mira::mcherry-MS2}* animals die as pharates. The ventral ganglion (VG)  
810 of these brains is frequently overgrown (arrows). Similar effects are seen with  
811 CrispR generated, homozygous *mira::mCherry::HA* larvae (not shown). **(B)** In  
812 fixed *w<sup>1118</sup>* brains Mira as well as its cargo Brat are diffuse in the cytoplasm of  
813 NB daughter cells and Deadpan (Dpn) staining is restricted to NB nuclei. **(B')**  
814 *BAC{mira::mcherry-MS2}* brains are not overgrown, Mira is sometimes more  
815 stable at the cortex in a daughter cell (arrowhead), but Dpn is normal. **(C)** In  
816 2x *BAC{mira::mcherry-MS2}* animals, Mira is strongly cortical in several NB  
817 daughter cells and so is Brat (arrowheads). Dpn is no longer restricted to NB  
818 nuclei but frequently found in clusters of smaller nuclei close to NBs  
819 suggesting that Mira is stabilized at the cortex and fails to release its cargo,  
820 inducing fate changes. mCherry is fused to the C-terminus of Mira which was  
821 shown to be required for cargo release (Fuerstenberg et al. 1998; Matsuzaki  
822 et al. 1998). Scale bars: 10 $\mu$ m.

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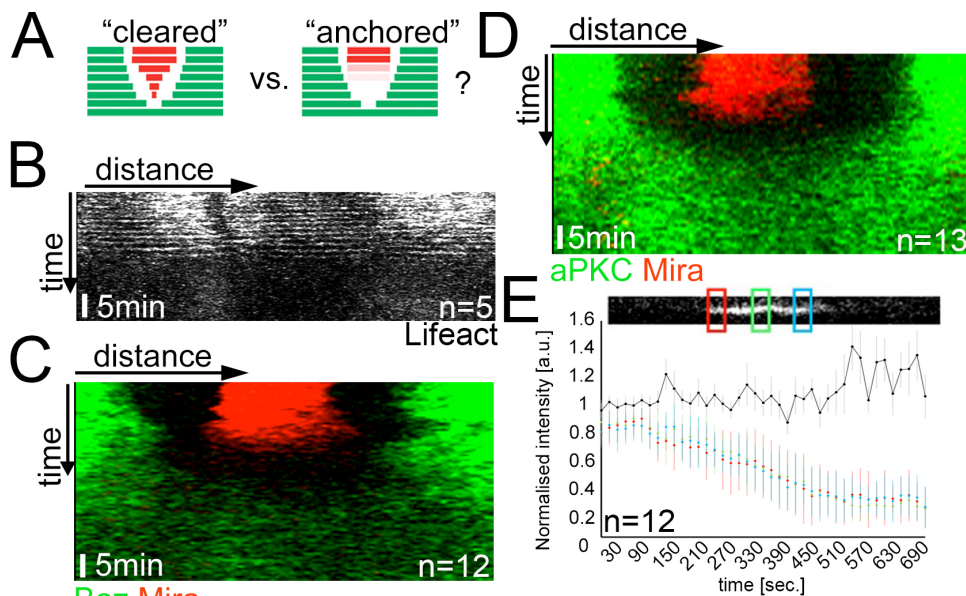
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Figure 1 supplement 3

825 **Figure 1 supplement 3.** Cortical Mira can be detected by antibody staining,  
826 in UAS-GFP-Mira overexpressing NBs and upon colcemid treatment, but not in  
827 interphase *mira*<sup>L44</sup> loss of function clones. **(A)** Antibody staining against Mira  
828 performed on different fixed isolated NBs in primary cell culture. In this assay  
829 Mira (red) is cortical in an interphase NB (judged by DAPI, blue). In prophase  
830 NBs, differently sized Mira “crescents” can be detected the ends of which are  
831 labeled by arrowheads. In a metaphase NBs Mira forms a crescent that  
832 appears larger than some of those seen in prophase NBs. **(B)** A living NB in  
833 primary cell culture expressing Mira::GFP driven by Mz1061. GFP signal is at  
834 the cortex in interphase, 29 min prior to NEB, GFP signal becomes cleared  
835 apically (arrowheads) until most of the cortex is cleared 1min prior to NEB. 5  
836 min after NEB a robust, larger crescent has formed. **(C)** Control brain treated  
837 with 50µM colcemid for 30min and stained for Mira. Over condensed  
838 chromatin in mitotic NBs demonstrates the effect of colcemid yet in all  
839 interphase NBs Mira remains at the cortex. **(D)** Fixed brain containing an  
840 interphase NB MARCM *mira*<sup>L44</sup> clone surrounded by control NBs. In the clone  
841 cortical Mira signal is gone (arrowhead) while present in a control interphase  
842 NB (arrow). Asterisks: mitotic NBs. Scale bar 10µm.

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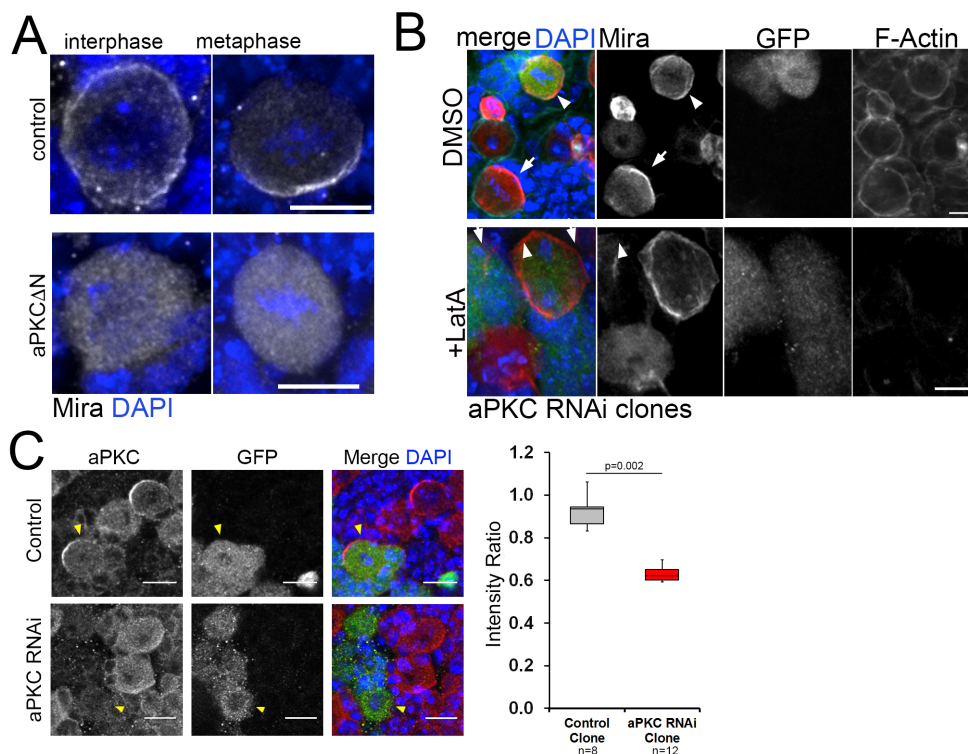


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Figure 2 supplement



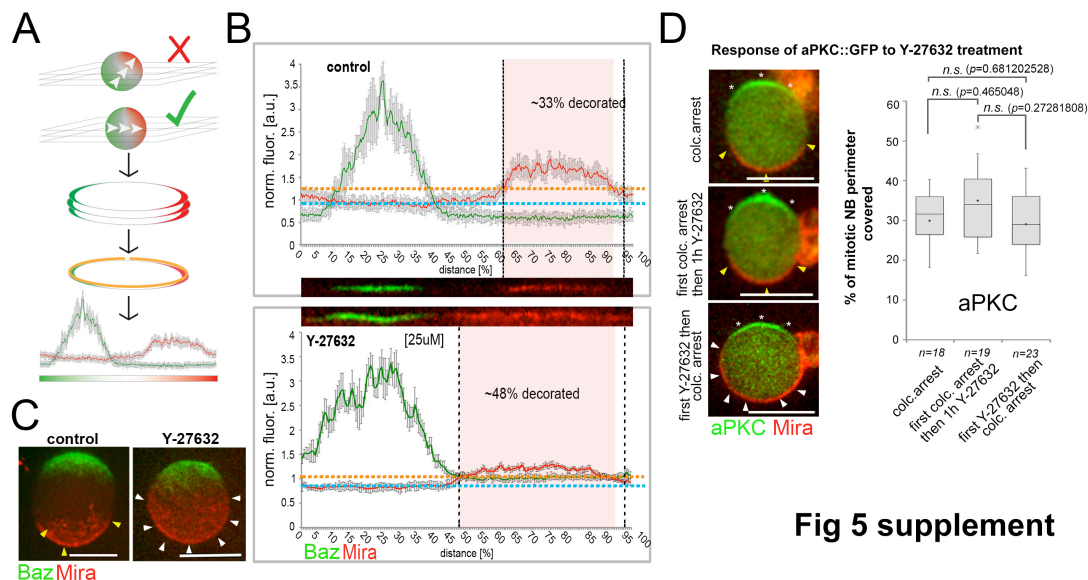
846 **Figure 2 supplement.** Mira falls homogenously off the cortex upon LatA  
 847 treatment, which is not driven by aPKC cortical displacement. **(A)** Schematic  
 848 depicting the expected kymograph profile for *clearing* versus *anchoring*. **(B-**  
 849 **D)** Kymographs of colcemid arrested NBs expressing Lifeact-Ruby, Baz::GFP  
 850 and Mira::mCherry or aPKC::GFP and Mira::mCherry (related to **Movie S4**)  
 851 upon the addition of 5 $\mu$ M LatA. The equatorial perimeter of the NB was  
 852 straightened out for each time point. Time scale bar: 5min. **(E)** Plot of Mira  
 853 decline upon LatA treatment of a colcemid arrested NB using 3 ROIs  
 854 (positions as indicated). Black line shows ratio ROI3/ROI2 over time, which  
 855 remains constant. Error bars: standard deviation. *BAC{mira::mcherry-MS2}*  
 856 was the source of Mira::mCherry. Scale bar: 10 $\mu$ m.  
 857  
 858  
 859



**Figure 3 supplement**

860  
 861  
 862 **Figure 3 supplement.** Effects of aPKC manipulation on Mira localization  
 863 under different conditions. **(A)** Mira antibody staining on whole mount brains  
 864 in a control NBs and in a NBs that expresses aPKC $\Delta$ N by *worniuGal4*. Control:

865 Mira is cortical in interphase (91% cortex, 9% cytopl., n=53), forms a  
 866 crescent in metaphase (93% crescent, 7% cytopl., n=15). aPKC<sup>ΔN</sup>: Mira is  
 867 cytoplasmic in interphase (85% cytoplasm, 15% cortex, n=40) and  
 868 metaphase (89% cytopl., 11% crescent, n=15). **(B)** aPKC RNAi expressing  
 869 flip out clones (GFP positive, arrowheads) and GFP negative control NBs  
 870 (arrows) treated with DMSO or 5μM LatA and stained with an antibody  
 871 against Mira and with Phalloidin to label F-Actin. In GFP negative mitotic  
 872 control NBs, Mira is in a crescent (DMSO) or cytoplasmic (LatA). Mira is  
 873 cortical in DMSO as well as LatA treated mitotic aPKC RNAi NBs (100%, n=5,  
 874 arrowheads). **(C)** aPKC is efficiently knocked down by RNAi in flip out clones.  
 875 aPKC RNAi NBs have significantly less aPKC. Scale bar: 10μm.  
 876



**Fig 5 supplement**

877

878

879 **Figure 5 supplement.** Standard used to quantify Mira crescent size. **(A)**  
 880 Schematic of workflow. NBs that have a polarity axis parallel to the imaging  
 881 plane are selected. 3-5 optical planes are collected covering 2-3μm of the  
 882 equator of the NB. **(B)** Fluorescence is normalized against the cytoplasmic  
 883 background and straightened line plots are derived from each section. The  
 884 average background and the average standard deviation is determined.  
 885 Signal: > avg. background plus two times the average standard deviation. **(C)**  
 886 3D projections of z-sections covering the entire NBs, ctrl vs. a NB that  
 887 polarized in the presence of 25μM Y-27632. **(D)** Quantification of aPKC::GFP

888 crescent size under the indicated conditions (unpaired ttest). Asterisks: aPKC;  
889 yellow arrowheads: normal sized crescents; white arrowheads: enlarged Mira  
890 crescents. *BAC{mira::mcherry-MS2}* was the source of Mira::mCherry. Scale  
891 bar: 15µm.

892

### 893 **Movie captions**

#### 894 **Movie S1**

895 Interphase cortical Miranda is removed at the onset of mitosis. Spinning disc  
896 confocal image of a neuroblast expressing Baz::GFP (red) and Mira::mCherry  
897 (green). For this and all subsequent videos maximum projection after a 3D  
898 Gaussian blur (FIJI, radius 8/.8/1) of 7 consecutive equatorial planes taken at  
899 0.4µm spacing is shown. Z-stacks taken every minute. Time stamp: hh:mm.

900

#### 901 **Movie S2**

902 Interphase cortical Miranda is removed at the onset of mitosis. Spinning disc  
903 confocal image of a neuroblast expressing aPKC::GFP (green) and  
904 Mira::mCherry (red). Z-stacks taken every minute. Time stamp: hh:mm.

905

#### 906 **Movie S3**

907 Interphase cortical Miranda is actin independent. Spinning disc confocal  
908 image of a neuroblast expressing Baz::GFP (red) and Mira::mCherry (green)  
909 showing a control division before 1µM LatA was added. Z-stacks taken ~every  
910 minute. Z-stacks taken every minute. Time stamp: hh:mm.

911

#### 912 **Movie S4**

913 Colcemid arrested NBs expressing aPKC::GFP and Mira::mCherry that were  
914 treated with 5µM LatA at the beginning of the recording at 16sec intervals.  
915 The cortex was straightened out and split at the apical pole such that  
916 aPKC::GFP appears right and left and Mira in the centre. Fluorescence profiles  
917 shown below. Note that Mira falls off homogenously from the cortex and  
918 becomes cytoplasmic at 5:36 (red arrowhead), while the detectable borders  
919 of cortical aPKC (green arrowheads) have not yet changed. Only from 7:12

920 onward aPKC rise above cytoplasmic levels where Mira was localized. Time  
921 stamp: mm:ss.

922

### 923 **Movie S5**

924 Myosin inhibition reversibly affects basal Mira anchoring in a polarized  
925 neuroblast. A colcemid arrested NB expressing Baz::GFP (green) and  
926 Mira::mCherry (red) in primary cell culture was treated with 20 $\mu$ M ML-7 which  
927 as washout when indicated. Left panel Baz::GFP, middle panel Mira::mCherry,  
928 right panel merge. Z-stacks taken every minute. Time stamp: mm:ss.

929

### 930 **Movie S6**

931 The effect of ML-7 on cortical Mira localization in mitosis can be delayed by  
932 overexpressing Sqh<sup>EE</sup>. Mira::mCherry NBs (ctrl) and Mira::mCherry NBs co-  
933 expressing Sqh<sup>EE</sup> (rescue) were co cultured in neighboring clots in the same  
934 dish and the effect of ML-7 on cortical Mira recorded. Z-stacks taken every  
935 two minutes. Time stamp: hh:mm.

936

### 937 **Movie S7**

938 Miranda remains at the cortex throughout the cell cycle in *apkc*<sup>k04603</sup> mutant  
939 NBs. Spinning disc confocal image of an *apkc*<sup>k04603</sup> mutant NB, labeled with  
940 nlsGFP (green) expressing Mira::mCherry (white). Z-stacks taken every  
941 minute. Time stamp: hh:mm.

942

### 943 **Movie S8**

944 Control *mira*<sup>mCherry</sup> allele generated by CrispR/Cas9. Mira localizes to the  
945 interphase cortex, from where it is cleared before NEB. Then Mira re-localizes  
946 to a larger crescent. Therefore this allele and Mira::mCherry (BAC rescue) are  
947 undistinguishable in terms of Mira dynamics. This control further shows that  
948 the MS2 binding site in the BAC rescue construct does not interfere with Mira  
949 cortical dynamics. Time stamp: hh:mm.

950

951

952 **Movie S9**

953 Phosphomutant S96A allele of Mira tagged with mCherry at the C-terminus.  
954 Mira localizes uniformly to the interphase cortex. Shortly before NEB, S96A is  
955 apically enriched, before being uniformly cortical after NEB and during  
956 division. Time stamp: hh:mm.

957

958 **Movie S10**

959 Phosphomimetic S96D allele of Mira tagged with mCherry at the C-terminus.  
960 S96D localizes to microtubules in interphase, but is asymmetric in mitosis in  
961 mitosis. Note the signal resembling subcortical microtubules in interphase  
962 converging at the apical pole. After NEB a basal crescent is detectable. At  
963 115:30 a z-stack spanning the entire NB was collected and the maximum  
964 projection is frozen. After this 50 $\mu$ M colcemid was added to reveal if  
965 Mira<sup>S96D</sup>::mCherry binds to the cortex. Next frozen frame: similar stack after  
966 30min in colc. Next frozen frame: 50 in colcemid – no cortical signal is  
967 detectable. Last frozen frame 65min in colcemid. Time stamp: mm:ss. Scale  
968 15 $\mu$ m.

969

970 **Movie S11**

971 Mira requires its BH motif for interphase cortical localization (see main text)  
972 and basal localization in mitosis. The BH motif in Mira has been deleted by  
973 gene editing and this Mira mutant tagged with mCherry at the C-terminus  
974 (*mira* <sup>$\Delta$ BHmCherry</sup>). Mira <sup>$\Delta$ BH</sup>::mCherry when homozygous is found on the  
975 interphase microtubule network and in the cytoplasm during mitosis. Time  
976 stamp: hh:mm.

977

978 **Movie S12**

979 200 $\mu$ M Y-27632 induced uniform cortical Mira in mitosis localizes  
980 independently of an intact actin network. A Baz::GFP and Mira::mCherry  
981 expressing NBs was cultured in the presence of 200 $\mu$ M Y-27632, arrested  
982 with colcemid. 5 $\mu$ M LatA was added after the first frame of the movie. LatA

983 induces loss of Baz asymmetry, yet Mira remains cortical. Z-stacks shown. Z  
984 stacks taken every 2min. Time stamp: hh:mm.

985

### 986 **Movie S13**

987 Colcemid arrested NBs expressing Baz::GFP and Mira::mCherry, treated with  
988 200 $\mu$ M Y-27632. Mira starts to become visible  $\sim$ 36min after Y-27632 addition  
989 in this example, but remains asymmetrically distributed, until LatA is added.  
990 Z-stacks shown. Z stacks taken every 2min. Time stamp: mm:ss.

991

### 992 **Competing interest declaration**

993 The authors declare no competing financial interests.

994

### 995 **Author contribution**

996 M.H., A.R., N.L. and J.J. designed and carried out experiments and  
997 interpreted the data and M.H., N.L. and J.J. wrote the manuscript that was  
998 agreed upon by all authors.