The endoplasmic reticulum is partitioned asymmetrically during mitosis before cell fate selection in proneuronal cells in the early *Drosophila* embryo

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ABSTRACT **Asymmetric cell division is the primary mechanism to generate cellular diversity, and it relies on the correct partitioning of cell fate determinants. However, the mechanism by which these determinants are delivered and positioned is poorly understood, and the upstream signal to initiate asymmetric cell division is unknown. Here we report that the endoplasmic reticulum (ER) is asymmetrically partitioned during mitosis in epithelial cells just before delamination and selection of a proneural cell fate in the early *Drosophila* embryo. At the start of gastrulation, the ER divides asymmetrically into a population of asynchronously dividing cells at the anterior end of the embryo. We found that this asymmetric division of the ER depends on the highly conserved ER membrane protein Jagunal (Jagn). RNA inhibition of *jagn* just before the start of gastrulation disrupts this asymmetric division of the ER. In addition, *jagn*-deficient embryos display defects in apical-basal spindle orientation in delaminated embryonic neuroblasts. Our results describe a model in which an organelle is partitioned asymmetrically in an otherwise symmetrically dividing cell population just upstream of cell fate determination and updates previous models of spindle-based selection of cell fate during mitosis.**

Monitoring Editor
Wallace Marshall
University of California, San Francisco

Received: Oct 4, 2016
Revised: Mar 28, 2017
Accepted: Mar 28, 2017

INTRODUCTION

A fundamental step in the development of a multicellular organism is the generation of cellular diversity. In the *Drosophila* embryonic CNS, this is accomplished by an intrinsic asymmetric cell division in which a proneural cell delaminates from the neuroepithelium and divides along the apical-basal axis to generate a ganglion mother cell and a self-renewing neuroblast (NB) cell (Doe, 1992; Technau *et al.*, 2006). The mechanism driving this asymmetric division is well conserved (Roegiers and Jan, 2004). The correct establishment of cell polarity is critical for cell fate selection, as mutational analysis in several organisms demonstrates that loss of cell polarity disrupts asymmetric divisions in the CNS and neuronal cell diversity (Jan and Jan, 2001; Bardin *et al.*, 2004).

Establishment of cell polarity relies on the correct positioning of cell fate determinants, including the evolutionarily conserved protein complex Bazooka/Par3, Par6, and atypical protein kinase C (aPKC; Kuchinke *et al.*, 1998; Petronczki and Knoblich, 2001; Rolls *et al.*, 2003). In embryonic models of neural development, establishment of apical-basal polarity regulates a rotation of the bipolar mitotic spindle during mitosis along the apical-basal axis, leading to delamination of the NB (Siegrist and Doe, 2006). In addition, proper apical-basal orientation of the spindle relies on the cortical adaptor protein Inscuteable (Insc; Kraut *et al.*, 1996). Changes in cellular organization before an asymmetric division are poorly understood, however, and the cue preceding these divisions and subsequent establishment of cell fate has yet to be identified.

The endoplasmic reticulum (ER) is the largest membrane-bound organelle in the cell and is responsible for many critical cellular

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E16-09-0690) on April 5, 2017.
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Abbreviations used: ER, endoplasmic reticulum; Jagn, Jagunal; NB, neuroblast.

functions, including lipid synthesis and protein secretion through the secretory pathway. The ER network during interphase consists of sheets close to the nucleus and tubules stretching out toward the periphery of the cell. During mitosis, the ER is highly dynamic, remodeling its structure to form large, sheet-like clusters as the cell enters mitosis and reorganizing its localization to the centrosomes during metaphase (Bobinnec *et al.*, 2003; Bergman *et al.*, 2015). ER-asymmetric partitioning has been reported in cells that are dividing asymmetrically, including the *Drosophila* larval neuroblast, the first divisions of the early *Caenorhabditis elegans* embryo, and the growing bud of *Saccharomyces cerevisiae* (Estrada *et al.*, 2003; Poteryaev *et al.*, 2005; Smyth *et al.*, 2015). Asymmetric ER partitioning has also been observed in muscle cell fate selection in the early ascidian embryo (Sardet *et al.*, 2007). However, the role of the ER in early embryonic asymmetric divisions has yet to be discovered. Whereas these changes in ER structure and localization during mitosis have been well documented (McCullough and Lucocq, 2005; Lu *et al.*, 2009), it is unclear what the asymmetric partitioning of the ER accomplishes and whether it contributes to cell fate determination.

To investigate the changes in cellular organization before and during the initial selection of cell fate, we focused on the first conventional cell division at the start of the midblastula transition in the early *Drosophila* embryo. *Drosophila* development begins with 13 synchronous, rapid syncytial nuclear divisions (Foe and Alberts, 1983). After these 13 divisions, the cellular blastoderm forms during a prolonged interphase of cycle 14, and each nucleus is encapsulated into a cell, transforming a one-cell multinucleated embryo into a multicellular embryo (Foe *et al.*, 1993; Schejter and Wieschaus, 1993). Once the cellular blastoderm has formed, local populations of cells termed mitotic domains divide asynchronously in a highly reproducible pattern and form the epithelial layers that eventually give rise to the different tissues of the adult fly (Foe, 1989; Campos-Ortega and Hartenstein, 1997). In addition, these mitotic domains are numbered according to when they enter mitosis 14. After mitosis 14 in the 15-cell cycle, the first asymmetric divisions of the NB occur in the embryo, providing an excellent platform to examine the changes that take place between symmetric and asymmetric divisions.

We focused our examination on mitotic division 14 after cellular blastoderm formation at the start of gastrulation, which is the division before the first NB delamination and subsequent asymmetric divisions. We observed a striking asymmetry of the ER during mitotic division 14 in a cell population that will eventually give rise to a domain of the *Drosophila* brain termed the anterior protocerebral ectoderm (Urbach *et al.*, 2003). We report that asymmetric ER division is restricted to this population of cells, occurs in a periclinal manner, and precedes the delamination of this cell population.

We also found that inhibition of Jagunal (Jagn), an evolutionarily conserved ER transmembrane protein, disrupted ER asymmetry and partitioning during mitosis. Jagn is known to facilitate membrane traffic to the subcortex of the oocyte and is necessary for oocyte growth and development (Lee and Cooley, 2007). In examination of Jagn localization at mitosis, we find that Jagn is partitioned asymmetrically at metaphase in this population of proneural cells. Furthermore, examination of delaminated NB in *jagn*-deficient embryos found defects in spindle alignment along the apical-basal axis. Taken together, these results demonstrate the novel finding that ER asymmetry during early development occurs before cell fate selection. This asymmetric partitioning relies on the ER transmembrane protein Jagn and represents an asymmetric event in symmetrically dividing cells before establishment of apical-basal polarity.

RESULTS

The ER is asymmetrically partitioned during mitosis 14 in mitotic domain 1

To examine ER organization and dynamics during conventional cell division before delamination and cell fate selection, we investigated cells during mitosis 14 at the start of gastrulation in the early *Drosophila* embryo. We focused our investigation on the first cellular domain to enter mitosis 14 and divide at the start of gastrulation, mitotic domain 1, which is located in two dorsolateral clusters at the anterior part of the embryo (Figure 1A). It is important to note that the cells found at this developmental stage are uniform in origin and have yet to differentiate. We generated a transgenic line expressing both the luminal ER marker protein disulfide isomerase fused to green fluorescent protein (Pdi-GFP) and the DNA marker His2Av fused to red fluorescent protein (H2-RFP) and imaged mitotic division 14 in domain 1 (Figure 1, B and C, and Supplemental Movie S1). We found that the ER established an asymmetric localization beginning at prometaphase, resulting in an unequal distribution of ER during cytokinesis. At the start of mitosis and entry into prophase, the ER is located symmetrically around the nuclear envelope. However, in prometaphase, the ER becomes more defined and concentrated at the nuclear envelope in an asymmetric pattern (Figure 1C and Supplemental Movie S1). This asymmetric localization persists during metaphase and anaphase, and during telophase, this unequal distribution of the ER is passed along to the newly formed daughter cells. This asymmetric partitioning of the ER was not observed in all dividing cells in mitotic domain 1; instead, it was restricted to ~17% (27 of 158) of the cells observed. Of importance, this number of ER-asymmetric cells in domain 1 is similar to the number of protoneuroblasts observed to delaminate from the neuroectoderm, ~16% (Urbach *et al.*, 2003).

To examine this asymmetric distribution quantitatively, we analyzed GFP intensity and tracking throughout mitosis. At the start of prometaphase, the cell was divided into two equal regions along the equatorial plane of the cell, and GFP intensity was normalized at prophase (Figure 1C, bottom, dotted yellow line). From our initial measurements at prometaphase, there was a significant difference in GFP intensity between the two domains. This difference was maintained throughout the stages of mitosis, and at cytokinesis, one daughter cell received a greater amount of GFP signal than the other daughter cell (Figure 1D).

To confirm our observations of ER asymmetry during cell division, we examined a second ER marker, Reticulon-like 1 (Rtnl1). The *Drosophila* Rtnl1 belongs to a family of membrane ER-shaping proteins that promote curvature of ER tubular structures (Voeltz *et al.*, 2006; Wakefield and Tear, 2006). Transgenic Rtnl1-GFP/H2-RFP embryos were imaged throughout mitosis in mitotic domain 1. Similar to our results with Pdi-GFP, Rtnl1-GFP also displayed an asymmetric localization (Supplemental Figure S1A). This result was also subject to our quantitative measurement of GFP intensity, and similar to Pdi-GFP, albeit to a lesser extent, there was a difference in GFP intensity between the two dividing daughter cells (Figure 1D). These results describe an unexpected asymmetry of the ER in a population of cells during mitotic division 14 before cell differentiation.

The ER divides symmetrically during division 14 in mitotic domains not associated with neuroblast development

We sought to determine whether this ER-asymmetric segregation is specific to mitotic domain 1, which gives rise to the anterior protocerebral ectoderm, or is seen in other cell populations at mitotic division 14. Therefore we investigated division 14 in mitotic domain 4, a posterior population of cells located at the tip of the germ band

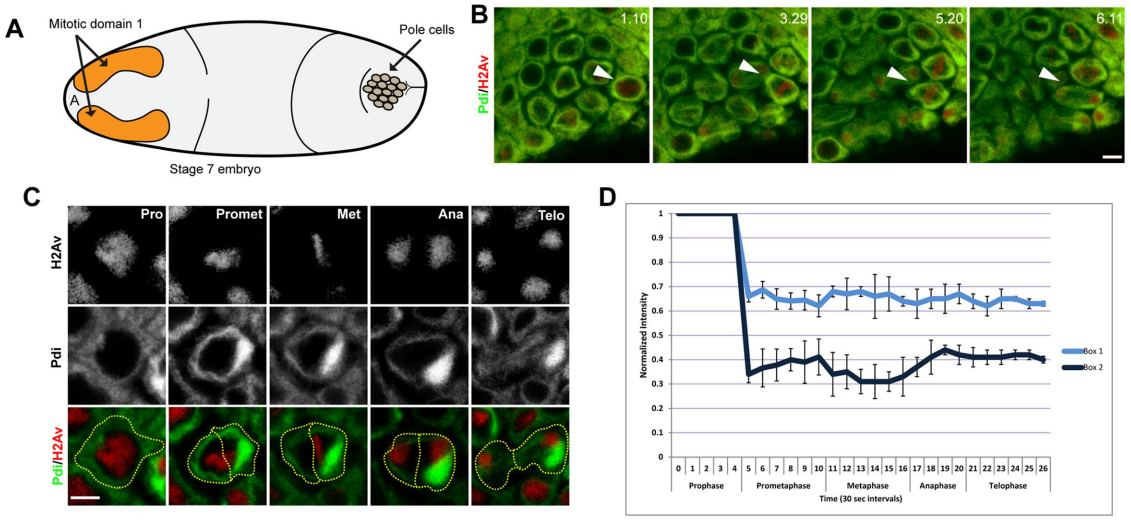

FIGURE 1: The ER displays asymmetric segregation during mitosis in mitotic domain 1. (A) Schematic of the location of mitotic domain 1 (orange) in a *Drosophila* embryo. The anterior axis of the embryo is marked A. (B) Transgenic *Drosophila* embryos expressing the ER marker Pdi-GFP (green) and the DNA marker H2Av-RFP (red) were imaged using scanning confocal microscopy at the start of gastrulation. A dividing population of cells in mitotic domain 1 (arrowheads) at low magnification at mitosis 14. (C) High-magnification images of a cell expressing ER marker Pdi-GFP undergoing mitosis in the first mitotic domain. Still images represent maximum intensity projection of the entire *Z*-stack. (D) Quantification of GFP intensity for Pdi-GFP through mitosis. The cell was divided into two regions, and the intensity of Pdi-GFP was measured (yellow dotted line in C, bottom). Each intensity point was measured at 30-s intervals from prometaphase to telophase. $N_{Pdi} = 8$ embryos imaged, $n_{Pdi} = 67$ mitotic events analyzed, $q_{Pdi} = 3$ computed repeats. Error bars represent SE. Scale bar, 5 μm.

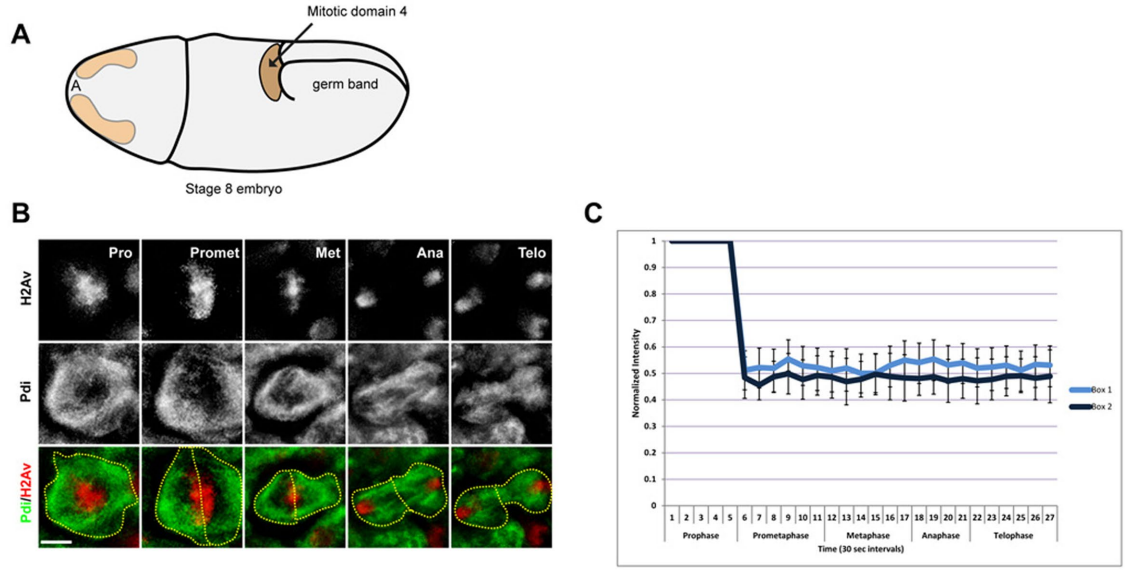

FIGURE 2: The ER does not display asymmetric segregation in mitotic domain 4. (A) Schematic of the location of mitotic domain 4 (brown) in a *Drosophila* embryo. Anterior axis is marked A. (B) Transgenic *Drosophila* embryos expressing the ER marker Pdi-GFP (green) and the DNA marker H2Av-RFP (red) were imaged using scanning confocal microscopy. Imaging occurred after the start of gastrulation during the onset of rapid germ band elongation. High-magnification images captured cells undergoing mitosis in mitotic domain 4. Images represent maximum intensity projection of the entire *Z*-stack. (C) Quantification of GFP intensity for Pdi-GFP through mitosis. The cell was divided into two regions, and the intensity of Pdi-GFP was measured (yellow dotted line in C, bottom). $N_{Pdi} = 5$ embryos, $n_{Pdi} = 21$ mitotic events analyzed, $q_{Pdi} = 3$ computed repeats. Error bars represent SE. Scale bar, 5 μm.

that gives rise to the anal pads of the adult fly (Figure 2A). Pdi-GFP/H2-RFP embryos were imaged during division 14 in mitotic domain 4 and subject to quantification of GFP intensity throughout mitosis. In contrast to mitotic domain 1, the ER displayed a symmetric division during mitosis in mitotic domain 4 (Figure 2B and Supplemental Movie S2). During prometaphase and into metaphase, the ER is evenly distributed around the mitotic spindle and aligned chromosomes. This distribution persists as the cell exits mitosis and results

Mitotic domain	Neuroblast population	Asymmetric ER partitioning observed	Percentage of ER asymmetric cells within a mitotic domain
1	Anterior	Yes	17 (27/158)
3	None; fuses with domain 1; forms preoral cavity	No	–
4	None; anal pads	No	–
5	Posterior (dorsal)	Yes	10.7 (8/75)
9	Posterior (ventral)	?	–
B	Central	?	–
21	None; ventral ectoderm parasegment boundaries	No	–
25	None; ventral ectoderm	No	–

The cell fate of the observed mitotic domains and whether asymmetric partitioning was observed during mitosis 14. Populations that give rise to NBs appeared to display cells that partitioned their ER asymmetrically. Domains 9 and B could not be observed due to the plane of division and the lack of a mitosis before delamination, respectively. Mitotic domains giving rise to NB populations were derived from fate-mapping experiments (Urbach *et al.*, 2003), and other descriptions of cell fates within mitotic domains are provided (Foe, 1989).

TABLE 1: Observations of ER-asymmetric partitioning in the mitotic domains during mitosis 14.

in symmetric ER partitioning to the daughter cells. Quantification of the GFP intensity throughout mitosis confirmed this symmetric division, showing no significant difference of the ER partition between the two newly formed daughter cells (Figure 2C). Symmetric ER distribution was also seen with our secondary ER marker, Rtnl1-GFP, during mitosis in mitotic domain 4 (Supplemental Figure S2, A and B). We conclude that ER partitioning in mitotic domain 4 during mitosis 14 occurs symmetrically.

Urbach *et al.* (2003) examined the development of the procephalic neuroectoderm and found that a small percentage of cells in mitotic domain 1 are fated to become the anterior brain NB. On the basis of our observations of asymmetric ER partitioning in mitotic domain 1, we hypothesized that other mitotic domains that give rise to NB populations will also experience an asymmetric ER partitioning. We examined mitotic domains that generate brain NBs (1, 5, 9, and B). We found that ER-asymmetric partitioning at mitosis 14 also occurs in domain 5 (Table 1), which develops into the posterior (dorsal) population of brain NBs. In addition, 10.7% (8 of 75) of the cells in domain 5 were observed to experience an ER partitioning during cell division. We also attempted to observe mitotic domains 9 and B, which develop into the posterior (ventral) and central NB populations, respectively. However, domain B is a nondividing population of cells at mitosis 14 (Foe, 1989), and domain 9 experiences an apical-basal rotation event at mitosis 14, which precluded examination of ER partitioning (Foe, 1989; Siegrist and Doe, 2006). On the basis of these observations, we conclude that asymmetric ER partitioning occurs in mitotic domains that give rise to the brain NB.

RNA interference inhibition of the ER transmembrane protein Jagn disrupts asymmetric division of the ER

Our results of asymmetric ER partitioning in mitotic domain 1 shared a close proximity to the cortex during division. This led us to hypothesize that a cortical factor associated with the ER is responsible for this asymmetry. A possible candidate is the ER membrane protein Jagn. Lee and Cooley (2007) identified and characterized Jagn as an integral ER membrane protein necessary for oocyte growth by clustering of the ER to the lateral cortex during vitellogenesis. The necessity of Jagn in development is clear, as homozygous *jagn* mutants die during the first- and second-instar larval stages, and a *jagn* germline deletion results in small eggs that fail to develop (Lee and Cooley, 2007). To investigate the role of Jagn in ER localization during gastrulation, we developed an RNA interference (RNAi) strategy in which double-stranded RNA (dsRNA) corresponding to Jagn was injected in the anterior region of Pdi-GFP/H2-RFP embryos at the start of cellularization (Figure 3A). After completion of cellularization, time-lapse confocal imaging containing Jagn dsRNA was performed in mitotic domain 1. After Jagn RNAi knockdown, entry into mitosis proceeded normally; however, the ER did not establish an asymmetric localization at prometaphase (Figure 3B and Supplemental Movie S3). Many of the cells showed a pronounced delay at metaphase (Supplemental Figure S3), and as the cell exited mitosis, the ER was partitioned symmetrically. Although a small percentage of the cells displayed transient asymmetric ER localization at metaphase, this localization was not maintained, and as the cell exited mitosis, the ER moved back toward the plane of division (Figure 3B, arrows). Quantitation of the GFP intensity throughout mitosis showed that with inhibition of Jagn, there was no significant difference in ER partitioning between daughter cells (Figure 3C). On the basis of these observations, we conclude that Jagn is necessary for correct asymmetric partitioning of ER in mitotic domain 1.

Jagn displays a cortical localization and asymmetrically segregates during mitosis

Because Jagn is necessary for asymmetric division of the ER, we sought to examine the subcellular localization of Jagn during mitosis in mitotic domain 1. We examined localization using a Jagn-Venus transgenic line, with Venus fused in-frame with Jagn at the C-terminus. This line has been shown to have a fully functional Jagn protein that recapitulates normal subcellular localization within the ER in the oocyte (Lee and Cooley, 2007). Jagn-Venus was imaged at the start of mitosis 14 in domain 1, and at prophase, the majority of Jagn was found throughout the cytoplasm (Figure 4A). At prophase, Jagn localizes along the cell cortex and is spread throughout the cytoplasm. During prometaphase, there is a shift in Jagn localization toward one half of the cell. By metaphase, Jagn localization is asymmetrically distributed to one half of the cell (Figure 4A, arrowhead). As the sister chromatids segregated during anaphase, Jagn localized along with one of the chromatids (Figure 4A, arrowhead), whereas the other segregating chromatid appeared to have no Jagn present (Figure 4A, arrow). At telophase, when the set of chromatids is decondensing, the majority of Jagn is found in one of the two daughter cells. We also measured whether this asymmetric partitioning of Jagn colocalized with the asymmetric localization of ER in these cells (Figure 4B). Here we injected Jagn-Venus transgenic embryos with

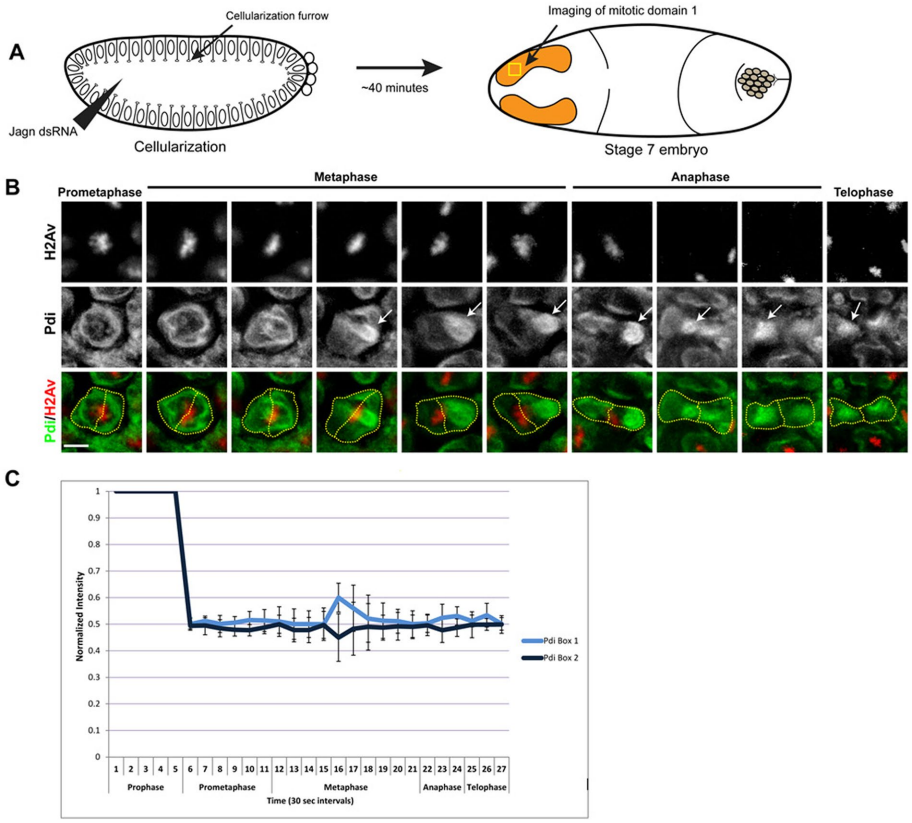

FIGURE 3: Inhibition of Jagn disrupts asymmetric ER partitioning. (A) Schematic of ds Jagn RNA injection strategy and imaging of *Drosophila* embryo. Jagn dsRNA was injected in the anterior part of the Pdi-GFP and H2Av-RFP embryo early in cellularization, allowing the dsRNA to be incorporated into every cell before the ~6000 nuclei are encapsulated. The injected embryo was imaged at the start of gastrulation (yellow box) in mitotic domain 1. (B) High-magnification images of a cell during mitosis in the presence of Jagn dsRNA. Early in mitosis, the ER aligned asymmetrically along the metaphase plate (arrows). Images represent maximum intensity projection of entire *Z*-stack. Scale bar, 5 μm. (C) Quantification of Pdi-GFP injected with Jagn RNAi through mitosis. For each slice in the *Z*-stack, the intensity of ER was taken and normalized to the intensity of the ER for the whole cell during prophase (not shown). Graph illustrates the average intensity of ER localization from prometaphase to telophase. NPdi = 4 embryos, nPdi = 20 mitotic events analyzed, qPdi = 3 computed repeats.

the lipophilic dye $DiIC_{18}$, which labels ER membrane in the early embryo (Parry, 2005). We imaged the embryo at the start of gastrulation in domain 1 using superresolution microscopy. Figure 4B is a single-plane image of a cell during mitosis, with Jagn (green) and ER membrane (red) localized both at the poles and along the perispindle region. There is a large pool of Jagn asymmetrically localized toward one spindle pole during metaphase (Figure 4B, arrow). Of interest, Jagn appears to localize just below the bulk of the ER membrane at the pole close to the mitotic spindle, suggesting an association with the microtubule network (see *Discussion*). As the cell exits mitosis, there is a population of Jagn that remained along the perispindle region through anaphase to cytokinesis (Figure 4B, arrowhead). This is in contrast to what was observed using conventional confocal microscopy of Jagn localization, which displayed a complete asymmetric distribution during metaphase (Figure 4A). Measurements of fluorescence intensity from our superresolution images throughout mitosis showed that both Jagn and ER had an asymmetric colocalization at one of the spindle poles (Supplemental Figure S4). Our superresolution imaging of Jagn localization during mitosis showed that Jagn along with ER membrane is distributed asymmetrically at metaphase and that Jagn localization along the perispindle region persists through mitotic exit. This asymmetric distribution of Jagn during cell division supports the conclusion that Jagn is necessary for asymmetric partitioning of the ER.

Jagn mutants display defects in spindle orientation in embryonic neuroblast

Examination of the pattern of NB formation in the early *Drosophila* embryo showed that cells in mitotic domain 1, after delaminating from the epithelial layer, are fated to become the anterior procephalic NB (Urbach *et al.*, 2003). Current models report a spindle rotation event during delamination that is coordinated with the distribution of asymmetric localization of cell fate determinants along the apical-basal axis in the early *Drosophila* embryo (Siegrist and Doe, 2006; Knoblich, 2008). Here we sought to investigate whether Jagn displayed any defects in spindle rotation and alignment in delaminated NB. To examine whether embryos deficient for Jagn possess any defects in this process, we immunostained homozygous $jagn^{Q21X}$ embryos at stage 11 (after cell cycle 15) with the apical cell polarity marker aPKC and tubulin to mark the position of the mitotic spindle. $jagn^{Q21X}$ is a presumptive null allele, as germline clones (GLCs) display severe defects in oocyte growth, and homozygous $jagn^{Q21X}$ die during the second larval stage (Lee and Cooley, 2007). During mitosis in asymmetrically dividing NBs at stage 11, aPKC forms a crescent at the apical cortex at metaphase (Doe *et al.*, 1991; Rolls *et al.*, 2003). In control NB, at metaphase, the spindle rotates and is aligned along the apical basal axis (Figure 5A); however, in *jagn* embryonic NB, spindle orientation and alignment are defective. In *jagn*-mutant NB, the mitotic spindle displays a range of orientation defects, including aligning perpendicular to the apical-basal axis. To quantify this defect, we measured the angle between a line through the bipolar spindle and a line bisecting the center of the apical aPKC crescent, with the correct orientation defined as 0° and the most severe defect defined as 90° (Figure 5B). In control NBs, the measured angle was found not to exceed 30° in 100% of neuroblasts, with 82% of spindles rotating <10° (n = 36; Figure 5C). However, in $jagn^{Q21X}$ embryos, only 58% rotated within 10°, with an appreciable subset of 38% displaying rotation defects >30° (Figure 5C). Of this subset, 9% of the neuroblasts displayed severe rotation defects of 90°, where the spindle was misaligned with the apical and basal crescents. It is important to note that aPKC (Figure 5A) and the basal cell polarity marker Prospero localized properly during mitosis in *jagn* mutants (unpublished data), suggesting that alignment defects are not due to defects in apical-basal polarity. In addition, bipolar spindle morphology during mitosis appeared normal in both control and mutant NBs (Figure 5A). From these data, we conclude that Jagn is required for proper spindle orientation in NBs and suggest a role for vesicle transport and recycling in spindle alignment during asymmetric division.

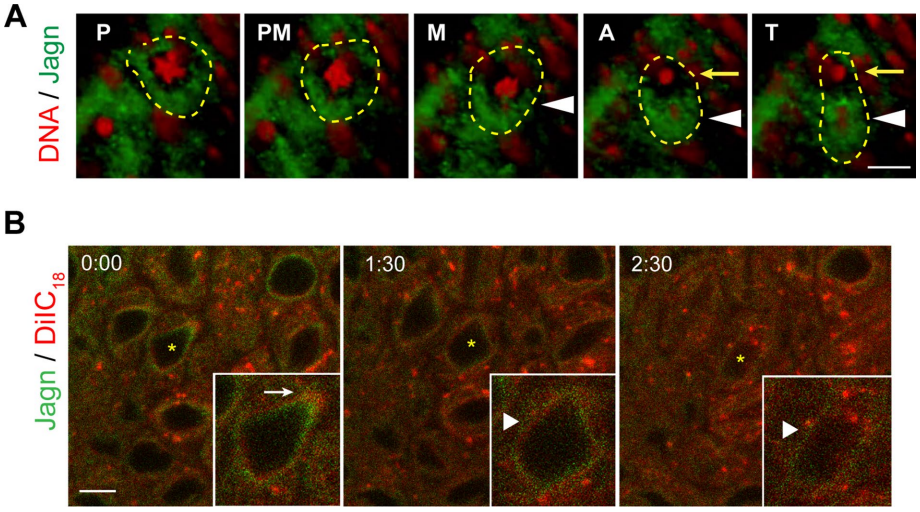

FIGURE 4: Jagn localizes to the cortex early in mitosis. (A) Jagn-Venus transgenic embryos were imaged during mitosis in domain 1 at the start of gastrulation. High-magnification images of a cell going through mitosis 14 (yellow dotted line). At prophase (P), Jagn (green) localizes throughout the cytoplasm, and in prometaphase (PM), as the chromosomes (red) are condensing, Jagn localization begins to shift to one side of the cell. During metaphase (M), Jagn is localized asymmetrically on one side of the cell (arrowhead). At anaphase (A), as the chromosomes are moving toward opposite ends of the dividing cell, Jagn is found asymmetrically partitioned to one of the daughter cells. In telophase (T), as the chromosomes are decondensing, Jagn is found in only one of the daughter cells (arrowhead), whereas the other daughter cell contains no Jagn (arrow). Scale bar, 5 μm. (B) Jagn colocalizes with the asymmetrically partitioned ER during metaphase. Jagn-venus (green) transgenic embryos were injected with the lipophilic dye $DiIC_{18}$ (red) to label the ER membrane and imaged using superresolution microscopy. A single plane was imaged during metaphase at the start of gastrulation of dividing cells in mitotic domain 1. Jagn displays an asymmetric distribution coinciding with asymmetric ER partitioning at one spindle pole (arrow). Insets, high magnification (2×) following a cell through late mitosis (asterisk). In superresolution images, there appears to be Jagn localized at the poles and along the perispindle region (arrowhead). Scale bar, 8 μm.

DISCUSSION

Here we showed a previously uncharacterized asymmetric division of the ER during mitosis 14 in a symmetrically dividing cell population in mitotic domain 1 at the start of gastrulation. An important aspect of this ER-asymmetric partitioning is that it occurs just before the delamination event in which one of the two daughter cells becomes the NB (Foe, 1989; Urbach *et al.*, 2003). We also demonstrate that the highly conserved ER transmembrane protein Jagn is necessary for this asymmetric division of the ER. This asymmetric division can be disrupted by RNAi knockdown of Jagn. Furthermore, Jagn displays an asymmetric localization at metaphase and displays NB defects in apical-basal spindle rotation. Our combined data suggest a role for Jagn in mediating ER/cortical interactions for the asymmetric partitioning of the ER. Our findings highlight an asymmetric partitioning of an essential organelle before the cell fate selection of the NB, a neural stem cell, and suggest additional mechanisms involved in the establishment of stem cell populations.

The role of Jagn in asymmetric ER partitioning at the cortex

Our data demonstrate that the evolutionarily conserved ER membrane protein Jagn is necessary for proper asymmetric partitioning of the ER during cell division (Figure 3B). The study by Lee and Cooley (2007) categorized Jagn's role in the reorganization of the ER in the growth of the *Drosophila* oocyte. Jagn contains four transmembrane domains and a dilysine motif at the extreme C-terminus, highlighting its role as an ER-resident protein and suggesting a role in retention of ER proteins via retrieval from post-ER membranes (Cosson *et al.*, 1998). Receptors that contain dilysine motifs are associated with type I COP membrane transport, suggesting that the N-terminus of Jagn may associate with an ER retention protein or receptor responsible for the transport of these cell fate determinants or factors involved in spindle orientation. In examination of embryonic NB in *jagn*-deficient embryos, the cell fate markers aPKC and Prospero were able to localize normally (unpublished data), indicating that Jagn does not play a role in the transport of cell fate determinants. In support of a role in spindle orientation, a clinical study on patients with severe congenital neutropenia (SCN) identified a new SCN allele, the human homologue of Jagn (JAGN1; Boztug *et al.*, 2014). The study performed a global proteomic analysis of JAGN1 and found that JAGN1 associates with various COP I components and also with tubulin, suggesting a role as ER/microtubule adaptor. Future work involving the connection between the microtubule network and the ER may help shed light on the role of Jagn in mitotic spindle positioning.

Asymmetric positioning of the ER and its role in development

Our data demonstrate an asymmetric partitioning of the ER during mitosis before cell fate selection in the early *Drosophila* embryo. Specifically, we find that the ER is positioned unevenly along the perispindle region and between the centrosome and cortex at metaphase, resulting in an unequal partitioning of the ER at cytokinesis. This finding of asymmetric positioning of the ER is not specific to *Drosophila* development but has been reported in other systems, primarily in ascidian embryos, but also in *Xenopus* and mouse oocytes (Mehlmann *et al.*, 1995; Chang *et al.*, 2004; Patalano *et al.*, 2006). Of interest, at the eight-cell stage in ascidian embryos, asymmetric divisions establish the germline and muscle tissues and are governed by the centrosome attracting body (CAB; Patalano *et al.*, 2006). The CAB is composed of an assemblage of cortical ER and maternal mRNAs and is surrounded by a mitochondrial-rich cytoplasm on the posterior side of the embryo between the centrosomal asters and the cortex. The CAB localizes with the cell polarity factors aPKC, Par-6, and Par-3/Bazooka and has been shown by micromanipulation experiments to be necessary for proper asymmetric divisions (Nishikata *et al.*, 1999). In prior studies examining development, both ascidians embryos and *Xenopus* oocytes also displayed cortical ER associated with RNAs and a mitochondrial cloud involved in the inheritance of germplasm (Chang *et al.*, 2004; Nakamura *et al.*, 2005). In addition, several other studies involving ER localization in a variety of organisms displayed a clustering of the ER at the cortex primarily during meiosis II (Sardet *et al.*, 1992; Mehlmann *et al.*, 1995; Terasaki *et al.*, 2001; Lee and Cooley, 2007). Most of these studies focused on the ER being responsible for localized Ca^{2+} release necessary for maturation, but based on recent work, the ER may be playing a greater role in development and cell fate selection.

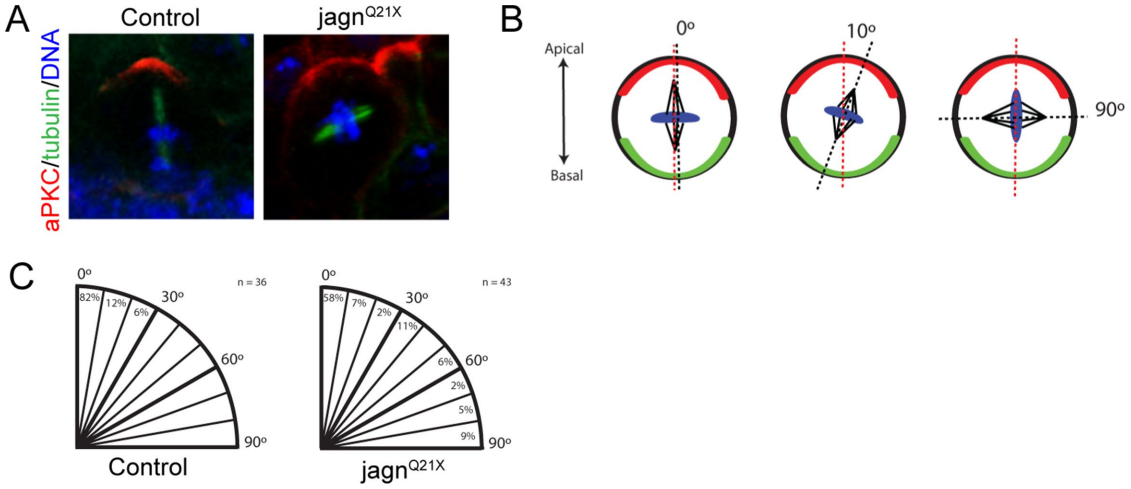

FIGURE 5: The *jagn* mutant NBs display mitotic spindle orientation defects along the cortical polarity axis. (A) Wild-type control (Oregon-R) or *jagn*^{Q21X} mutant NBs imaged at mitosis 15 after delamination from the epithelial layer. Embryos were stained for the apical marker aPKC (red), tubulin (green), and DNA (blue). Homozygous *jagn*-mutant embryos display defects in apical-basal mitotic spindle alignment at metaphase of mitosis 15 (right). Scale bar, 5 μm. (B) Quantification of mitotic spindle alignment in NBs relative to apical (red) and basal (green) polarity. The wild-type phenotype is defined here as a 0° angle formed by the spindle (dashed black line) and the apical/basal axis (dashed red line). Slight misalignment is shown with an angle of 10°, as well as the most severe misalignment with an angle of 90°. (C) Quantification of mitotic spindle orientation of wild-type control and *jagn*^{Q21X} mutant NBs. *jagn*^{Q21X} embryos have a greater angle of incidence with respect to the apical-basal axis than controls.

Planar polarity may govern ER asymmetric partitioning

The mechanism that governs asymmetric division consists of two events: establishment of cell polarity and spindle alignment along this polarity axis (Lu and Johnston, 2013). Studies in *Drosophila* have led to two models of polarity control with respect to asymmetric divisions in NB, apical-basal polarity, and planar polarity: apical-basal polarity is responsible for embryonic NB development, and planar polarity is responsible for larval sensory organ precursor cells (Jan and Jan, 2001). Our examination of mitotic division 14 in proneuronal cells of the CNS and the observation of asymmetric partitioning of the ER suggest a planar polarity mechanism in NB development. Mitotic division 14 and ER-asymmetric partitioning occur before the delamination of these proneuronal cells in mitotic domain 1 at the corresponding interphase of cycle 15 (Urbach *et al.*, 2003). During division 14, the apical-basal axis has yet to be established, and the spindle orientation factor Insc is not expressed until division 15 (Kraut *et al.*, 1996), suggesting that apical-basal polarity is not driving ER-asymmetric partitioning in the proneuronal cells in domain 1. The division preceding delamination in the early embryo appears to be similar to the epithelial expansion seen in the developing mouse skin, where cells divide within the epithelial plane until the division orientation switches to an apical-basal mechanism later in development (Lechler and Fuchs, 2005). This switch from planar divisions to apical-basal divisions depends on Notch signaling (Williams *et al.*, 2011), and a next step in this work is to see whether ER asymmetry also relies on Notch signaling.

Asymmetric ER partitioning and its involvement with mitotic spindle rotation events

It is well established that the ER shares a close association with the microtubule network during interphase (Terasaki *et al.*, 1984, 1986), and studies have shown that the ER is closely aligned with the bipolar spindle at mitosis (McCullough and Lucocq, 2005; Lu *et al.*, 2009; Bergman *et al.*, 2015). There is no known mechanism of ER involvement in mitotic spindle assembly and alignment, although a study indicates a regulatory relationship between the ER Ca^{2+} sensor STIM1 and the microtubule +TIP-binding protein EB1 during mitosis (Smyth *et al.*, 2012). This may indicate a possible link between the ER and the correct positioning of the mitotic spindle. Further support for this idea comes from studies of the ER-comprised CAB in ascidian embryos, which showed that proteins associated with CAB are responsible for correct spindle positioning during muscle cell fate selections (Prodon *et al.*, 2010).

Taken together, our data in the context of previous studies suggest two possible roles for ER-asymmetric partitioning. Either the ER is partitioned asymmetrically to provide necessary mRNAs for proper cell fate selection or ER-asymmetric partitioning is responsible for the correct spindle positioning during cell division. It is important to note that these two possibilities may not be mutually exclusive, as both could be occur as necessary for asymmetric division and proper establishment of tissue layers. Future studies investigating coordination of mitotic spindle events and ER movement through these divisions during development will further elucidate the molecular mechanisms required for asymmetric organelle division and generation of cellular diversity.

MATERIALS AND METHODS

Drosophila strains

We used the following fly strains: Oregon R (wild type), H2Av-RFP;Pdi-GFP (Bergman *et al.*, 2015), Rtnl1-GFP (Morin *et al.*, 2001), Jagn-Venus (Lee and Cooley, 2007), Jagn^{Q21X} (Lee and Cooley, 2007), and Dr[Mio]/TM3, P{w[+mC] = GAL4-twi.G}2.3, P{UAS-2xEGFP}AH2.3, Sb[1] Ser[1], P{hs-Gal4} (Bloomington *Drosophila* Stock Center). All strains were kept at 25°C and reared according to standard protocols (Ashburner, 1989).

Live-embryo analysis

Embryos were staged according to criteria established by Campos-Ortega and Hartenstein (1997). Pdi-GFP; H2Av-RFP embryos were prepared for microinjection and time-lapse scanning confocal

microscopy as previously described (Riggs *et al.*, 2007). For live imaging of mitotic domains, embryos were positioned perpendicular to the coverslip with the dorsal aspect facing the coverslip. Microinjection of embryos was performed with ~0.1 µl of Jagn dsRNA injected into the dorsoanterior region of the embryo just after the start of cellularization (~160–170 min after egg deposit). For imaging of the ER, the lipophilic dye $DiIC_{18}$ Stain (ThermoFisher; Parry, 2005) was microinjected into the dorsoanterior region of the embryo. DiIC18 was solubilized in soybean oil as previously described (Mehlmann *et al.*, 1995; Parry, 2005).

RNAi synthesis

PCR primers targeting Jagn were previously designed by the University of California, San Francisco, version 2 *Drosophila* RNAi library (Goshima *et al.*, 2007). T7 promoter sequence was added to the 5′ end of the forward and reverse primers. A PCR using the T7-modified primers was performed on isolated *Drosophila* genomic DNA to yield a 548–base pair product. The in vitro transcription reactions were performed using a T7 MEGAscript kit (Ambion).

Live imaging, processing, and analysis

Live imaging of mitotic domains was performed on a Zeiss LSM 710 upright scanning confocal microscope using a 63× oil objective and 2× field zoom. All live imaging was done under identical laser and gain settings, and *Z*-stacks were taken at predicted patch locations (Foe, 1989). Each *Z*-stack consisted of 25 × 1-µm steps that encompassed the entire depth of the cell monolayer. Maximum Intensity projections were created and used to create movies and still images. Superresolution imaging was performed on a Leica TCS SP8 X White Light Laser confocal microscope with Hybrid spectral detectors and HyVolution. These images were acquired and processed with the Leica LAS X software (version 3.1.1).

LSM *Z*-stack files from the LSM710 microscope were fed into a custom CellProfiler/MATLAB pipeline (Carpenter *et al.*, 2006). CellProfiler program was programed to identify interphase/prophase cells based on shape, size, and H2Av-RFP intensity. Baseline intensity of GFP ER marker was taken at prophase. At metaphase, two regions were created along the metaphase plate, and GFP intensity was measured within each region and compared with the baseline intensity measurement. Intensity data were loaded into MATLAB for compiling and analysis. Normalized intensities >50.1% were grouped as Box 1, and normalized intensities ≤50% were grouped as Box 2. Line graphs were generated from the data set, and a two-tailed unequal-variance Student's test was performed between Box 1 and Box 2 to determine significance at each time point.

We used ImageJ (v2.0) to examine quantitatively the superresolution image regarding ER and Jagn colocalization at the poles. A region of interest (ROI) was created around each pole of the cell, and area, mean fluorescence, integrated density and six background means were measured. Total corrected cellular fluorescence (= integrated density – [area of ROI × mean fluorescence of background]) was used to calculate fluorescence intensity to account for varying ROI areas (John and McCloy, 2014). Fluorescence intensity was measured for Jagn and DiI separately in both poles, and line graphs were generated from the data set using Excel.

Embryo fixation and immunohistochemistry

Fixed imaging of *Drosophila* embryos was performed on $jagn^{Q21X}$/TM3, Twi-GFP neuroblasts during cell cycle 15. Homozygous $jagn^{Q21X}$ embryos were selected based on absence of TM3, Twi-GFP balancer. Four hours after fertilization, embryos were fixed using paraformaldehyde (16% wt/vol) and immunostained with mouse anti-aPKC (1:500; Santa Cruz Biotechnology), and mouse E7 anti–β-tubulin (1:500; Developmental Studies Hybridoma Bank). DNA was imaged using 4′,6-diamidino-2-phenylindole (1:5000; Sigma-Aldrich). Neuroblasts were identified based on size (<~10 µm).

To quantify mitotic spindle orientation, the angle measure between a line bisecting apical aPKC crescents and the line created by the mitotic spindle was measured. In this way, proper orientation was defined as close or equal to 0°, and the most severe defect was defined as a 90° rotation.

ACKNOWLEDGMENTS

We thank Annette Chan (Cell and Molecular Imaging Center, San Francisco State University) and David Castaneda-Castellanos (Leica) for technical expertise and use of imaging equipment. We also thank Miri VanHoven for critical reading of the manuscript and Lynn Cooley for providing fly stocks. Funding of this work was through National Institutes of Health Minority Biomedical Research Support R25-GM059298Z (A.E. and A.A.), National Science Foundation Center for Cellular Construction Award 1548297 (N.G.), the California State University Program for Education and Research in Biotechnology (S.B.), and National Science Foundation CAREER Award 1553695.

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