Regulation of cortical stability by RhoGEF3 in mitotic Sensory Organ Precursor cells in Drosophila

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ABSTRACT

In epithelia, mitotic cells round up and push against their neighbors to divide. Mitotic rounding results from increased assembly of F-actin and cortical recruitment of Myosin II, leading to increased cortical stability. Whether this process is developmentally regulated is not well known. Here, we examined the regulation of cortical stability in Sensory Organ Precursor cells (SOPs) in the Drosophila pupal notum. SOPs differed in apical shape and actomyosin dynamics from their epidermal neighbors prior to division, and appeared to have a more rigid cortex at mitosis. We identified RhoGEF3 as an actin regulator expressed at higher levels in SOPs, and showed that RhoGEF3 had in vitro GTPase Exchange Factor (GEF) activity for Cdc42. Additionally, RhoGEF3 genetically interacted with both Cdc42 and Rac1 when overexpressed in the fly eye. Using a null RhoGEF3 mutation generated by CRISPR-mediated homologous recombination, we showed using live imaging that the RhoGEF3 gene, despite being dispensable for normal development, contributed to cortical stability in dividing SOPs. We therefore suggest that cortical stability is developmentally regulated in dividing SOPs of the fly notum.

KEY WORDS: Actin, Cell division, Cortical stability, Drosophila, RhoGEF3

INTRODUCTION

Epithelia function as protective and selective barriers between the external world and the body interior. In proliferating epithelia, cells adopt various shapes and dimensions at interphase: cells can be elongated in columnar and pseudostratified epithelia, or be flat in squamous epithelia. Despite these morphological differences, cells adopt at mitosis a spherical shape (Cadart et al., 2014; Lancaster et al., 2013; Maddox and Burridge, 2003; Ramanathan et al., 2015; Son et al., 2015; Stewart et al., 2011). This mitotic cell rounding is important for the efficient formation of a bipolar spindle and organization of a metaphase plate (Champion et al., 2017; Lancaster et al., 2013). Thus, upon mitosis, epithelial cells push on their neighbors as they round up. In columnar epithelia, mitotic cells can escape compression forces exerted by neighboring cells by dividing at the apical surface (Lee and Norden, 2013). However, in cuboidal epithelia facing a rigid apical substrate, such as the fly notum that is covered by a rigid pre-cuticle, mitotic cells round-up by pushing on their neighbors. Thus, increased cortical rigidity at mitosis may contribute to proper spindle geometry in cells growing in a mechanically constrained environment (Cadart et al., 2014; Cattin et al., 2015). At the molecular level, increased cortical rigidity at mitosis involves the cortical recruitment of MyoII and assembly of F-actin at the cell cortex downstream of the Rho-kinase (Maddox and Burridge, 2003) and mitotic kinases (Ramanathan et al., 2015; Rosa et al., 2015). In Drosophila, a RhoGEF known as Pebble (Pbl; Ect2 in mammals) acts as a Cdc42 GEF to regulate the formation of an isotropic actin cortex (Oceguera-Yanez et al., 2005; Rosa et al., 2015). Additionally, Moesin and its activating kinase Slik are also required for proper cortical rigidity and cell rounding (Carreno et al., 2008; Kunda et al., 2008). Thus, the mechanisms regulating cortical rigidity at mitosis and the relevance of mitotic rounding for optimal division geometry are now well established. Because most growing epithelia comprise different cell types, cell-specific regulation of mitotic rounding might exist. Whether mitotic rounding is developmentally regulated remains to be investigated.

The developing notum of Drosophila is an excellent model to study in vivo epithelial cell division and the role of mitotic spindle orientation in morphogenesis and cell fate (Bosveld et al., 2016; Gho and Schweisguth, 1998; Schweisguth, 2015). The pupal notum is a single-layered epithelium that produces the dorsal thorax of adult flies. This epithelium comprises two types of cell, the sensory organ cells and the epidermal cells. Epidermal cells divide with a random orientation (Besson et al., 2015; Bosveld et al., 2016; Gho et al., 1999). By contrast, Sensory Organ Precursor cells (SOPs) divide asymmetrically along the anterior-posterior axis of the fly body to produce an anterior pIIb cell and a posterior pIIa cell (Gho et al., 1999; Gho and Schweisguth, 1998). The orientation of the mitotic spindle is regulated by Frizzled-mediated Planar Cell Polarity (PCP) signaling (Bellâché et al., 2001a; David et al., 2005; Gho and Schweisguth, 1998; Gomes et al., 2009; Roegiers et al., 2001), whereas the binary pIIa/pIIb decision depends on the unequal segregation of two Notch regulators, Numb and Neutralized (Le Borgne and Schweisguth, 2003; Rhyu et al., 1994; Schweisguth, 2015). Thus, SOPs respond to PCP cues and orient its spindle in response to these global cues in face of more local compression forces, e.g. resulting from the division of neighboring epidermal cells. Considering the importance of mitotic rounding for cells dividing in a crowded environment, we wondered whether the dynamics of actin and myosin might be regulated in a SOP-specific manner.

Here, we found that SOPs and epidermal cells differed in actomyosin dynamics at interphase and that SOPs appeared to be more circular at mitosis than epidermal cells. We identified RhoGEF3 as a protein expressed at higher levels in SOPs and showed that
RhoGEF3 had \textit{in vitro} GEF activity for Cdc42. Moreover, RhoGEF3 was found to genetically interact with Cdc42 and Rac1 in a gain-of-function assay. We generated a null allele of the \textit{RhoGEF3} gene and observed that the activity of RhoGEF3 gene was largely dispensable for viability and development. However, loss of \textit{RhoGEF3} activity increased cortical instabilities in SOPs, suggesting that RhoGEF3 contributed to cortical stability in mitotic SOPs.

**RESULTS**

\textbf{Apical shape and actomyosin dynamics in SOPs}

While studying the polar distribution of Par6 and Baz in the single-layered epithelium that will form the dorsal thorax (Besson et al., 2015), we noticed that SOPs could be recognized from other non-SOP epithelial cells by their reduced apical area and their concave edges, leading to a characteristic inward-curving shape (Fig. 1A,A'). To measure the extent to which the apical area is concave, we used ‘solidity’ as a shape descriptor (‘solidity’ measures the ratio between the area of the apical cortex region and the convex hull of the shape; see diagram in Fig. 1B). Using the cell contours extracted from the time-lapse movies reported in Besson et al. (2015), we characterized the shape of the apical surface in SOPs and epidermal cells at interphase and found a significant difference in ‘solidity’ between these two types of cells prior to mitosis (Fig. 1B). Since epithelial cells are in a dynamic mechanical equilibrium at the level of their apical junction (Heisenberg and Bellaïche, 2013; Lecuit et al., 2011), this difference in cell shape suggested differences in mechanical properties between SOPs and their neighbors.

Within each cell, a contractile actomyosin meshwork generates active forces. High contractile activity along a given cell edge results in edge straightening and shortening. Conversely, increased contractility of medial actomyosin produces a centripetal flow associated with inward pulling forces exerted on cell edges (Heisenberg and Bellaïche, 2013; Martin et al., 2010, 2009; Martin and Goldstein, 2014). Accordingly, the observed inward-curving of the SOP edges indicated...
cell-specific differences in actomyosin dynamics between SOPs and their neighboring epidermal cells. To test this possibility, we examined the distribution of F-actin, using the Actin Binding Domain (ABD) of Moesin (Moe) fused to mCherry (Cherry-MoeABD), and Myosin II (MyoII), using a MyoII-GFP reporter. Live imaging of F-actin revealed an accumulation of F-actin at the apical surface of SOPs (Fig. 1C-C″). This observation confirmed earlier findings showing that F-actin localized differentially in SOPs and epidermal cells to promote the formation of microvilli in SOPs and in their progeny cells (Rajan et al., 2009). Here, we found that MyoII was recruited to the apical medial cortex, together with F-actin, in SOPs (Fig. 1C-C″). Moreover, contractile pulses and foci of MyoII were observed at the apical medial cortex, together with F-actin, in SOPs (Fig. 1C-C′′). Of note, no changes in supracellular junctional cables. Thus, our data revealed a change in mostly junctional in neighboring non-SOP cells and appeared to form (not shown). We conclude that adoption of the SOP fate led to cell-specific differences in actomyosin organization prior to asymmetric cell division.

**Actomyosin distribution in dividing SOPs**

We next studied the distribution of actomyosin in mitotic SOPs. Upon entering into mitosis, SOPs became spherical and this cell rounding correlated with the redistribution of actomyosin all around the cell to presumably produce a rigid cortex at prometaphase (Founounou et al., 2013; Lancaster et al., 2013) (Fig. 2A-D″). At anaphase, asymmetric cytokinesis was observed, with the plasma membrane ingressing more rapidly on the basal side as described earlier (Founounou et al., 2013; Guillot and Lecuit, 2013; Herszterg et al., 2013; Morais-de-Sá and Sunkel, 2013). Additionally, mitotic SOPs appeared to be less deformable than mitotic epidermal cells: live imaging indicated that SOPs were more circular at mitosis than epidermal cells (circularity values measured within the plane of the epithelium: 0.941±0.004 in SOPs versus 0.902±0.021 in epidermal cells; t-test, P=4×10⁻⁶). This suggested that mitotic SOPs might more efficiently push on their neighbors than mitotic epidermal cells. This might in turn suggest that cortical rigidity at mitosis could be higher in SOPs than in epidermal cells.

**RhoGEF3 is expressed at higher levels in SOPs**

We next investigated how cortical rigidity might be regulated in SOPs. A previous study has shown that the accumulation of F-actin and MyoII at the cell cortex in mitosis required the activity of a formin, Diaphanous (Dia), acting downstream of Cdc42 (Rosa et al., 2015). This finding raised the possibility that increased cortical rigidity in SOPs might result from increased activity of Cdc42 and/or of its downstream effectors. Interestingly, RNAseq analysis of pools of SOPs and epidermal that had been individually microdissected from fixed nota showed that a 1.8-fold increase in RhoGEF3 transcript levels in SOPs (P=0.004; K.M., unpublished). Despite its name, the RhoGEF3 gene may encode a GTPase Exchange Factor (GEF) for Cdc42/Rac, rather than for Rho (Greenberg and Hatini, 2011). Indeed, its closest mammalian ortholog, known as ARHGEF4 or ASEF, was shown to display GEF activity towards Cdc42 and Rac, but not Rho (Kawasaki et al., 2007, 2003, 2000). Moreover, analysis of the formation of multicellular capsules around parasitoid wasp eggs in fly larvae identified a requirement for Dia, Cdc42/Rac and RhoGEF3, whereas the activities of Rh1 and of its GEFs Pebble and RhoGEF2 were dispensable (Howell et al., 2012). These data are therefore consistent with RhoGEF3 acting as a Cdc42/Rac GEF, possibly...
upstream of Dia, for the regulation of actin in immune cells. We therefore decided to investigate further the role of RhoGEF3 in SOPs.

To further study the expression of the RhoGEF3 protein in the notum, we used BAC recombineering to generate a RhoGEF3-GFP transgene. The RhoGEF3 gene encodes multiple isoforms and because it is not known which of these isoforms are expressed in the pupal notum, we inserted GFP at the non-conserved C-terminus of RhoGEF3 that is shared by all predicted isoforms to produce RhoGEF3-GFP (Fig. 3A,B). Western blot analysis of larval brain-disc complexes indicated that both long and short isoforms of RhoGEF3 were present in these tissues (Fig. 3E). Furthermore, consistent with our RNAseq data, we found that RhoGEF3-GFP was present in all cells of the pupal notum at a low level and that SOPs exhibited higher levels of RhoGEF3-GFP accumulation (Fig. 3C,C’). At mitosis, RhoGEF3-GFP distributed all around the cortex (Fig. 3D). Thus, RhoGEF3 is expressed at higher levels in SOPs.

Our RNAseq analysis of SOPs identified several other actin regulators, including the Daam gene, one of the six formin family genes of Drosophila (3.2-fold increase in mRNA levels in SOPs, \(P=2\times10^{-5}\); K.M., unpublished). Using a Daam\(^{GFP}\) knock-in allele produced by CRISPR-mediated Homologous Recombination (HR), we confirmed that Daam was expressed at higher levels in SOPs and, like Dia, localized all around the cortex in mitotic SOPs (Fig. S1A-D’). While these expression and localization data suggested that Daam might regulate cortical stability in mitotic SOPs, the silencing of the Daam gene had no detectable effect on cell shape and cortical stability (data not shown). We therefore focused our analysis on the role of RhoGEF3 in cortical stability.

**RhoGEF3 has in vitro Cdc42 GEF activity**

We first studied the GEF activity of RhoGEF3 using an *in vitro* GEF assay. The activity of a 60 kDa fragment of RhoGEF3 that is present in all isoforms and which encodes the predicted GEF domain (RhoGEF3\(^{EK\delta}\)) (Fig. 4A) was produced in *E. coli* as a GST-fusion protein, purified and tested against Cdc42, Rac1 and RhoA. As a negative control, we used a version of RhoGEF3 that harbored mutations at conserved residues that are known to be important for the GEF catalytic activity of Lcp/p115 RhoGEF (Dubash et al., 2007) and GEF-H1 (Cullis et al., 2014). Specifically, the conserved E526 (numbering as in RhoGEF3-PA) was mutated into a Lysine (K) as in GEF-H1 (Cullis et al., 2014) and the conserved K677 and N726 residues were mutated into Alanine (A) as in Lcp/p115 RhoGEF (Dubash et al., 2007). The triple mutant protein used as a negative control in the GEF assay was referred to here as RhoGEF3\(^{KAA}\) (Fig. 4A). We used a fluorescent nucleotide analog (mant-GTP) to follow nucleotide exchange on purified Cdc42, Rac1 and RhoA. Since the binding of mant-GTP to the nucleotide binding pocket of a GTPase results in fluorescence increase (Leonard et al., 1994), an increase in fluorescence intensity indicated nucleotide exchange by the GTPase. Using this *in vitro* assay, we found that RhoGEF3\(^{EKN}\), but not RhoGEF3\(^{KAA}\), displayed GEF activity towards Cdc42, but not towards Rac or Rho (Fig. 4B-D). This result indicated that RhoGEF3 may act as a GEF for Cdc42.

**RhoGEF3 genetically interacts with Cdc42 and Rac1**

To further investigate *in vivo* the GEF activity of RhoGEF3, we generated transgenic flies expressing under the control of the UAS-Gal4 system two GFP-tagged versions of the long RhoGEF3 isoform (RhoGEF3-PL): a wild-type version (RhoGEF3\(^{EKN-GFP}\)) and a version created by CRISPR-mediated Homologous Recombination (HR), referred to here as RhoGEF3\(^{KAA-GFP}\). RhoGEF3\(^{EKN-GFP}\) localized at the apical cortex when expressed in the dorsal cells of wing imaginal discs under the control of the ap-Gal4 driver line. Additionally, expression of active RhoGEF3 led to increased F-actin levels along the lateral...
membranes (Fig. 5A-B″). This lateral accumulation of F-actin appeared to correlate with a shortening of cell height and formation of epithelial folds in the dorsal part of the wing pouch (data not shown). By contrast, the catalytically dead version of RhoGEF3, RhoGEF3KAA-GFP, localized along both apical and lateral membranes and had no effect on F-actin distribution and epithelium shape (Fig. 5C-C″). These observations are consistent with the notion that RhoGEF3 regulates the organization of the actin cytoskeleton in a GEF-dependent manner.

We next addressed whether RhoGEF3 could act in vivo as a GEF for Cdc42, as suggested by the results of the in vitro GEF assay (Fig. 4). To do so, we performed genetic gain-of-function interactions in the compound fly eye. The wild-type (active) or mutant (inactive) version of GFP-tagged RhoGEF3 were expressed together with the wild-type versions of Cdc42, Rac1 or Rho1 using the UAS/Gal4 system. Expression of these three GTPases in the photoreceptor cells using the GMR-Gal4 driver produced relatively minor defects in the structure of the adult eye (Fig. 6A-D), with the strongest defects being seen with Rac1 (Harsharan et al., 1995; Nolan et al., 1998). Of note, Cdc42 and Rac1 were overexpressed at 18°C to minimize the temperature-dependent activity of Gal4, whereas Rho1 was expressed at 25°C. Likewise, expression of wild-type and mutant RhoGEF3 did not significantly alter the adult eye structure (Fig. 6E,I). However,
expressing wild-type RhoGEF3 together with Cdc42 or Rac1 led to a fully penetrant late pupal lethality that was associated with a very strong eye defect in pharate adults (Fig. 6F,G). These phenotypes appeared to be similar to those reported earlier for the eye-specific expression of activated Cdc42, Rac1 and Rho1 (Benlali et al., 2000; Rangarajan et al., 1999; Schenck et al., 2003) (in our hands, driving the expression of Cdc42V12, Rac1V12 and Rho1V14 using GMR-Gal4 at 18°C led to early pupal lethality). The genetic interactions observed between RhoGEF3 and Cdc42/Rac1 were dependent on the GEF activity of RhoGEF3 as flies co-expressing Cdc42 or Rac1 with RhoGEF3KAA-GFP showed either no defect (Cdc42; Fig. 6J) or much reduced interaction (Rac1; Fig. 6K). Finally, no genetic interaction was observed between RhoGEF3 and Rho1 (Fig. 6H,L). Together, these data support the view that RhoGEF3 might act in vivo as a GEF for Cdc42 and Rac1. Thus, our in vitro and in vivo results suggest that RhoGEF3 acts as a Cdc42 GEF. Whether RhoGEF3 has also GEF activity towards Rac1 in vivo remains to be clarified (see discussion).

**RhoGEF3 contributes to cortical stability**

We next investigated the function of RhoGEF3 in the developing notum. To do so, we generated a null allele by creating a large deletion at the RhoGEF3 locus using CRISPR-mediated HR (Fig. 3A). The resulting RhoGEF3KO mutant flies were semi-viable and fertile with no obvious developmental defects but appeared weak and survived poorly. In particular, no fate defects were observed in the bristle lineage (not shown). This indicated that the activity of the RhoGEF3 gene is largely dispensable for proper fly development. To look at the possible function of the RhoGEF3 gene in SOP asymmetric division, we performed live imaging on GFP-Baz pupae expressing a dsRNA targeting RhoGEF3 under the control of the pnr-Gal4 driver. Although the silencing of the RhoGEF3 gene had no effect on the...
posterior accumulation of GFP-Baz, or on the anterior-posterior orientation of the division (not shown), it resulted in increased solidity in SOP (Fig. 1B). This observation was consistent with a role for RhoGEF3 in actin dynamics in SOPs. Additionally, bleb-like cortical instabilities were observed upon silencing of RhoGEF3 (arrows). At least 15 SOPs per genotype were studied.

Fig. 7. RhoGEF3 contributes to cortex stability in mitotic SOPs.

(A-C) Cortical distribution of Numb-GFP (green) and aPKC (red) in dividing SOP (Sens, blue) in wild-type (A) and pnr>RhoGEF3RNAi (B,C) pupae at 16.5 h APF. Cortical instabilities were observed upon silencing of RhoGEF3 (arrows). The number of cortical instabilities at the anterior cortex that transiently disrupted the Numb crescent (see Movie 2). Time is in s and t=0 corresponds to the metaphase-anaphase transition. (D–D‴) Live imaging of Numb-GFP in a RhoGEF3RNAi SOP. Selected snapshots showing cortical instabilities at the anterior cortex (arrows). At least 15 SOPs per genotype were studied. (D–D‴) Live imaging of Numb-GFP in a RhoGEF3RNAi SOP. Selected snapshots showing cortical instabilities at the anterior cortex that transiently disrupted the Numb crescent (see Movie 2). Time is in s and t=0 corresponds to the metaphase-anaphase transition. (E) Quantification of the number of cortical instabilities (seen using Numb-GFP) in dividing SOPs. Cortical instabilities were scored blind in wild-type control (n=19), RhoGEF3RNAi (n=23) and diaRNAi (n=18) SOPs. The number of cortical instabilities increased upon the silencing of the dia and RhoGEF3 gene. The metaphase-anaphase transition was chosen as t=0 (red arrow).

(F) Quantification of the number of cortical instabilities seen using Spider-GFP (per cell and time interval of 8 s; time in s) in dividing SOPs. Because division time varied with genotype and most cortical instabilities were observed soon after NEB (which could be observed using Spider-GFP; see panels G–I), the NEB was chosen here as t=0 (red arrow). Cortical instabilities were scored blind in wild-type control (n=19), RhoGEF3RNAi (n=22), RhoGEF3KO (n=19) and diaRNAi (n=15) SOPs. The number of cortical instabilities increased upon loss of dia and RhoGEF3 activities. (G–I) Kymographs of dividing Spider-GFP SOPs. The silencing of dia (H) and RhoGEF3 (I) led to cortical instabilities during prometaphase (yellow arrows; the red dots indicate the nuclear Spider-GFP signal used to detect the onset of NEB; time interval is 8 s). In wild-type pupae, the cortex remained stable from NEB to anaphase in SOPs (G). Scale bars: 5 µm.
detected in wild-type SOPs at prometaphase (Fig. 7A). This therefore raised the possibility that RhoGEF3 contributes to cortical stability in SOPs. To better study the role of RhoGEF3 in cortical stability, we performed live imaging using Numb-GFP as a cortical marker. Because loss of dia has previously been reported to promote blebbing in dividing epidermal cells of the fly notum, we used dia as a positive control. In wild-type pupae, blebbing and/or cortical instabilities were observed basally in SOPs rounded up during prophase but were more rarely seen after nuclear envelope breakdown (NEB). By contrast, a significant number of cortical instabilities were scored at prometaphase in both dia^{RNAi} and RhoGEF3^{RNAi} pupae (Fig. 7D-E; scoring was performed blind). These instabilities were transient and NumbGFP segregated correctly into the anterior pIIb cell as in wild-type cells (Fig. 7D-D’; Movie 2).

Since Numb-GFP only marked the anterior cortex, we next used a gigamesh-GFP fusion protein, SpiderGFP, which marked the entire cortex. Low levels of SpiderGFP were detected in the nuclei during interphase and at prophase. This allowed us to precisely time the NEB. SpiderGFP movies were analyzed by scoring cortical instabilities in wild-type, dia^{RNAi}, RhoGEF3^{RNAi} and RhoGEF3^{3KD} mutant pupae (again, scoring was performed blind). The results showed that reduced dia and RhoGEF3 activities led to more frequent cortical instabilities after NEB in mitotic SOPs relative to control SOPs (Fig. 7F-I). We conclude that RhoGEF3 contributes to increased cortex stability in mitotic SOPs. Together, our results suggested that cortical stability at mitosis may be modulated in a cell-specific manner in the fly notum via the regulated expression of RhoGEF3.

DISCUSSION

An Ect2/Pbl-Cdc42-Dia pathway was shown earlier to regulate mitotic rounding in epidermal cells of the fly notum (Rosa et al., 2015). This pathway was proposed to regulate the assembly of an isotropic actomyosin via the lateral spreading of Par6, hence triggering a switch upon mitotic entry to a Dia-mediated actomyosin via the lateral spreading of Par6, hence triggering a switch upon mitotic entry from an Arp2/3-mediated actin nucleation process active at interphase to a Dia-mediated process (Rosa et al., 2015). Par6 spreads laterally at the posterior cortex in SOPs (Bellaïche et al., 2001b), but it is unclear whether a similar switch takes place in SOPs and whether additional regulatory processes might contribute to an isotropic actomyosin cortex. Here, we identified RhoGEF3 as a protein expressed at higher levels in SOPs. Using an in vitro assay, we found that a large 60 kDa fragment of RhoGEF3 encompassing the catalytic DH-PH domains could act as a Cdc42 GEF. Consistent with this, the co-expression of active RhoGEF3 and Cdc42 in photoreceptor cells, but not the expression of either one alone, led to pupal lethality and defective eye formation. Since similar phenotypes were observed upon expression of activated Cdc42, the strong synergy between RhoGEF3 and Cdc42 indicated that RhoGEF3 might act as an in vivo GEF for Cdc42. Additionally, both in vitro Cdc42 GEF activity and in vivo genetic interaction were abolished upon mutation of key catalytic residues in the DH-PH domain of RhoGEF3. Thus, our data strongly suggest that RhoGEF3 can act as a Cdc42 GEF, like its mammalian homolog (Kawasaki et al., 2007, 2003, 2000). While the large 60 kDa fragment of RhoGEF3 encompassing the catalytic DH-PH domains showed no detectable GEF activity towards human Rh and Rac in vitro, RhoGEF3 was found to also synergize in vivo with Rac1 (but not Rho1). Thus, whether RhoGEF3 can also act as a Rac GEF, as shown for its mammalian homolog (Kawasaki et al., 2007, 2003, 2000), will require further investigation. In support of this, a very recent study used a GST pulldown assay to test for direct molecular interaction between in vitro translated RhoGEF3 with GDP/GTP-loaded GTas. This analysis indicated that RhoGEF3 can bind GTP-loaded Rac1 and might also regulate Cdc42 (Nakamura et al., 2017). These findings are consistent with our genetic interaction data showing a strong synergy between RhoGEF3 and Cdc42/Rac1. While it is conceivable that RhoGEF3 might regulate the localization of GTP-bound Rac1 (Nakamura et al., 2017) or act downstream of Rac1 activation as an effector of GTP-bound Rac, it is also possible that our in vitro assay failed to detect the Rac1 GEF activity of RhoGEF3. In summary, Drosophila RhoGEF3 acts as a GEF for Cdc42 and possibly Rac1, but is not a Rho GEF.

RhoGEF3 was recently reported to regulate the distribution of F-actin in a wound healing assay in embryos (Nakamura et al., 2017). Here, RhoGEF3 was found in a gain-of-function assay to alter in a GEF-dependent manner the distribution of F-actin in wing epithelial cells. This therefore suggested that RhoGEF3 has the ability to regulate F-actin distribution, presumably via Cdc42 and/or Rac1. To further examine the in vivo function of RhoGEF3, we used CRISPR-mediated homologous recombination to create a null deletion allele of the RhoGEF3 gene. Our genetic analysis, however, revealed that the activity of RhoGEF3 is largely dispensable for asymmetric division of SOPs and more generally for proper development in Drosophila. Nevertheless, our live imaging analysis of cortical stability indicated that RhoGEF3 plays a non-essential role in the regulation of cortical stability in SOPs. Whether these cortical instabilities correlated with changes in Cdc42-dependent F-actin dynamics was not further examined. Based on these data, we speculate that RhoGEF3 contributes, in parallel to other essential Cdc42/Rac GEFs such as Ect2/Pbl (Rosa et al., 2015), to the activation of Cdc42 (and possibly Rac, but not Rho) in mitotic SOPs, thereby promoting the formation of a rigid cortex at mitosis. While this activity is non-essential for the maintenance of SOP asymmetry at mitosis, we speculate that this activity might contribute to stabilize the cortical domains of asymmetrically dividing SOPs in face of local deformations, thereby ensuring that SOPs divide asymmetrically along a stereotyped division axis independently of the behavior of their neighbors within a crowded environment (Cadart et al., 2014). This view is consistent with earlier studies showing that increased actin polymerization, downstream of the SRF transcription factor, is essential for spindle orientation and asymmetric division in the skin of the mouse embryo (Luxenburg et al., 2011). While the fly homolog of SRF was not detected as being upregulated in SOPs, several actin regulators, including RhoGEF3 and Daam, were expressed at significantly different levels in SOPs versus epidermal cells. Thus, we speculate that RhoGEF3 might be only one of several activities contributing to the SOP-specific changes in actomyosin dynamics. Future studies looking for such redundant activities might in turn further our understanding of the in vivo function of RhoGEF3 in the cell-specific regulation of cortical stability.

MATERIALS AND METHODS

Transgenes, genome engineering and flies

The RhoGEF3-GFP BAC transgene was generated using recombinering mediated gap-repair from the BAC CH322-144N20 (Venken et al., 2009, 2006). The sfGFP flanked by GVG linkers was fused in frame at the C-terminus of RhoGEF3. The resulting BAC was integrated at the 3xP3-RFP.attP/ZH-51D site. The pUAS-RhoGEF3^{EKN}-GFP and pUAS-RhoGEF3^{3AA}-GFP plasmids were generated in two steps. First, the EKNO-KAA mutations were introduced in the RhoGEF3-GFP BAC using BAC recombinering. Second, genomic fragments encoding the short isoforms, e.g. RhoGEF3-PB, were PCR-amplified from wild-type and mutated BACs and subcloned as an EcoRI-XbaI fragment into the pUAS-attB vector. The resulting UAS-RhoGEF3^{EKN}-GFP and UAS-RhoGEF3^{3AA}-GFP plasmids
were integrated at the PBac(y+attP-3B)VK0002-28E7 site. Cloning details are available upon request. Plasmid and BAC injection was performed by Bestgene (Chinno).

The Daam<sup>WT</sup> and RhoGEF3<sup>WT</sup> lines were generated using CRISPR-mediated HR. For each line, two gRNA oligonucleotides were cloned into pU6-Bbs1-chiRNA (Addgene #45946) as described in addgene.org/crispr/ OConnor-Giles/. Donor templates for HR were first produced by BAC recombineering in E. coli and then transferred into multicopy vectors as described in Venken et al. (2006). A BAC encoding Daam (CH321-32015) was used to introduce sfGFP flanked by GVG linkers, at position 1087 of Daam-PA, i.e. 27 amino acids before the C-terminus. This position was chosen as a poorly conserved region between fly species. Daam<sup>WT</sup> flies were viable with no phenotype. A partial deletion of the RhoGEF3 gene was produced in the RhoGEF3 BAC. The 3xP3-RFP selection marker (flanked by loxP sites) was produced by gene synthesis and inserted at the position of the deletion. Left and right homology arms flanking the target sites were 1.5 kb long. Proper HR was verified by genomic PCR.

Other stocks used in this study were: UAS-RhoGEF3dsRNA, par6-GFP, GFP-Baz, Numb-GFP (Couturié et al., 2013), Spider-GFP, neur-H2B-RFP, sqh-Cherry-MoeABD, sqh-MyoII-GFP, UAS-Cdc42, UAS-Cdc42 (BL-28873), UAS-Rac1 (BL-28874), UAS-Rho1 (BL-9390), GMR-Gal4, ap-Gal4 and tub-Gal80<sup>°</sup>. Pictures of adult flies were taken on a AxioZoom V16 microscope (Zeiss, Jena, Germany).

**Live imaging and image analysis**

Live imaging of staged pupae and quantitative image analysis of Baz and Par6 asymmetry were performed as described earlier. Live imaging of mitotic SOPs was performed using a 63×/NA 1.4 objective (PL APO, D27; Leica Microsystems, Wetzlar, Germany) on a Zeiss LSM780 microscope. SOPs were identified using a Histone2B-RFP expressed under the control of a near cis-regulatory module (near-H2B-RFP) or based on the polar distribution of Numb-GFP. For shape analysis (solidity), GFP-Baz movies were segmented as described in Besson et al. (2015). Cell contours were converted to Fiji polygon ROI and solidity values were calculated over a time interval preceding mitosis (~14-15 h APF) using the corresponding Fiji plugin. Solidity values were represented as boxplots. For circularity at mitosis, segmented GFP-MoeABD movies were analyzed using the corresponding Fiji plugin. Cortical instabilities were scored blind in 4D (x,y,z,t) movies.

**Immunostainings**

Staged larval and pupal were dissected and stained following standard procedures. Primary antibodies were: rabbit and goat anti-GFP (1:1000; Abcam ab6673), mouse anti-Cut (1:500; clone 2B9, Developmental Studies Hybridoma Bank), guinea pig anti-Senseless (Nolo et al., 2000) (1:3000; a kind gift from H. Bellen, Baylor College of Medicine, Houston, USA), rabbit anti-aPKC (1:1000; sc216, Santa Cruz Biotechnology) and rabbit anti-Dia (Afsfar et al., 2000) (1:1000; a kind gift from S. Wasserman, UCSD, San Diego, USA). F-actin was detected using atto-647 phalloidin (Sigma-Aldrich).

**Western blot**

GFP-tagged RhoGEF3 was studied by western blot analysis using brain-disc complexes dissected from third instar larvae (five larvae per well). Protein extracts were prepared in 0.5% Triton buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 0.5 mM DTT, 1× protease inhibitor cocktail EDTA-free from Roche) and loaded on 4-20% precast Miniprotein TGX gels for SDS-PAGE. Proteins were transferred onto 0.2 µm Nitrocellulose membranes (BioRad). HRP-coupled anti-GFP antibodies (1:5000; Abcam, ab6663) were used to detect the different RhoGEF3-GFP isoforms with SuperSignal WestFemto (ThermoFisher Scientific).

**Protein production and in vitro GEF assay**

The fragments encoding the C-terminal part of wild-type (EKN) and mutant (KAA) RhoGEF3-PA (513 amino-acids) were PCR-amplified from the RhoGEF3 BAC (CH322-144N20) and cloned into the pGEX6P-2 plasmid (the EKN-to-KAA mutations were introduced in the BAC CH322-144N20 using BAC recombineering; cloning details available upon request). Following sequencing, plasmids were introduced in the BL-21 Rosetta strain for protein production. Recombinant proteins were purified on Glutathione sepharose beads (Amersham Bioscience). The RhoGEF3<sup>EKN</sup> and RhoGEF3<sup>KAA</sup> fragments were released from the beads by proteolytic cleavage using the prescision protease (GE Healthcare). Protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). The GEF activity of the purified RhoGEF3 fragments (0.8 µM) was tested using a fluorophore-based GEF assay that measures the uptake of the N-methylanthraniloyl-GTP (mant-GTP) by purified GT-Pases (2 µM) that were provided in the RhoGEF Exchange Assay Kit (Cytoskeleton Inc., Denver, USA) used for this assay (Leonard et al., 1994). We followed the manufacturer’s protocol for its 96-well plates. Fluorescence increase was measured on an Infinite M200 PRO spectrophotometer (Tecan, Mannedorf, Switzerland).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

Supplementary information available online at http://bio.biologists.org/lookup/doi/10.1242/bio.026641.supplemental

**References**


Supplementary Figure

Figure S1: SOP-specific expression of a GFP-tagged Daam generated by CRISPR-HR

A) Genomic organization of the Daam locus showing only two transcript isoforms (RA and RB). Exons are shown as boxes (ORF in black). The point of insertion of the GFP (green) at the Daam locus is indicated. All isoforms are tagged. The position of the deletion in the RhoGEF3\(^{KO}\) flies produced by CRISPR-HR is indicated: most of the sequence encoding the RhoGEF3-PA was deleted. The 3xP3-RFP selection marker was inserted at the position of the deleted segment.
B) Domain structure of Daam-PA, encoded by the RA transcript isoform (A). The GTPase Binding Domain (GBD, orange) and the Formin Homology domains (FH2, magenta; FH3, blue) are shown. GFP (green) was fused at the C-terminus.

C-D’) Daam-GFP (green) was detected at higher levels in SOPs (Senseless, red in C; Cut, red in D). Daam-GFP was detected at the apical cortex prior to division (C,C; aPKC, blue) and all around the cortex together with Dia (blue in D) at mitosis (D’).

**Supplementary Movies**

**Movie 1: Live imaging of MyoII**

Live imaging of GFP-tagged MyoII (green; time in min) in cells of the pupal notum. MyoII was mostly medial-apical in the SOP (at the center) and junctional in epidermal cells. Pulses and foci were observed in SOPs (note the cell shape deformation associated with the MyoII pulses).
**Movie 2: Live imaging of NumbGFP in wild-type and RhoGEF3RNAi SOPs**

Live imaging of GFP-tagged Numb in wild-type (left) and RhoGEF3RNAi (right) SOPs. Cortical instabilities (arrow) were more frequently observed upon silencing of the RhoGEF3 gene.