A Cdc42-regulated actin cytoskeleton mediates Drosophila oocyte polarization

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SUMMARY

Polarity of the Drosophila oocyte is essential for correct development of the egg and future embryo. The Par proteins Par-6, aPKC and Bazooka are needed to maintain oocyte polarity and localize to specific domains early in oocyte development. To date, no upstream regulator or mechanism for localization of the Par proteins in the oocyte has been identified. We have analyzed the role of the small GTPase Cdc42 in oocyte polarity. We show that Cdc42 is required to maintain oocyte fate, which it achieves by mediating localization of Par proteins at distinct sites within this cell. We establish that Cdc42 localization itself is polarized to the anterolateral cortex of the oocyte and that Cdc42 is needed for maintenance of oocyte polarity throughout oogenesis. Our data show that Cdc42 ensures the integrity of the oocyte actin network and that disruption of this network with Latrunculin A phenocopies loss of Cdc42 or Par protein function in early stages of oogenesis. Finally, we show that Cdc42 and Par proteins, as well as Cdc42/Par and Arp3, interact in the context of oocyte polarity, and that loss of Par proteins reciprocally affects Cdc42 localization and the actin network. These results reveal a mutual dependence between Par proteins and Cdc42 for their localization, regulation of the actin cytoskeleton and, consequently, for the establishment of oocyte polarity. This most likely allows for the robustness in symmetry breaking in the cell.

KEY WORDS: Cdc42, aPKC, Par-6, Bazooka, Actin, Polarity, Oogenesis, Axis formation, Drosophila

INTRODUCTION

Cell polarity is essential in all living organisms for many developmental and cellular processes, including differentiation, proliferation and morphogenesis. Cell polarity establishment and maintenance are therefore fundamental processes that involve several key players, many of them highly conserved between different tissues of the same organism and in different species. Cell polarity relies on the distinct localization of cellular components, such as proteins or lipids, to specific sites within the cell. Their localization is mediated by the polarized transport of these components or of mRNA precursors.

The Par proteins are conserved key players in cell polarity and maintenance and were first identified in Caenorhabditis elegans. Whereas symmetry breaking and thus axis establishment in the C. elegans embryo are determined by sperm entry, which triggers actin cytoskeleton and microtubule dynamics (Motegi et al., 2011; Munro et al., 2004), the anteroposterior and dorsoventral axes of the future Drosophila melanogaster embryo are established already during oogenesis (Brendza et al., 2000; Cha et al., 2002; Johnstone and Lasko, 2001; Theurkauf et al., 1993; Tian and Deng, 2008; Vanzo et al., 2007; Zimyanin et al., 2008). The Drosophila oocyte develops in an egg chamber consisting of the oocyte and 15 interconnected nurse cells, surrounded by a follicular epithelium. The oocyte is determined by the accumulation of oocyte-specific factors, which requires the microtubule network (Tian and Deng, 2008). Oocyte-specific factors and microtubule minus ends first accumulate at the anterior of the prospective oocyte, then translocate to the posterior of the cell (Huynh and St Johnston, 2000). This anterior-to-posterior translocation does not occur in par-1, bazooka (baz; the Drosophila par-3 ortholog), par-6 or atypical protein kinase C (aPKC) null mutants (Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b). The role of the Par proteins in regulating oocyte polarity and development is maintained throughout oogenesis, as par-1 and baz hypomorphs cause fully penetrant polarity defects, including mislocalization of axis-determining oskar (osk) mRNA (Doerflinger et al., 2010; Shulman et al., 2000; Tomancak et al., 2000). Par-6, aPKC and Baz localize to the anterolateral cortex of the oocyte, whereas Par-1 localizes to the posterior cortex (Goldstein and Macara, 2007). As a result, the Par proteins form complementary cortical domains that are maintained by mutual antagonism (Benton and St Johnston, 2003; Doerflinger et al., 2010; Hurov et al., 2004; Tian and Deng, 2008; Vaccari and Ephrussi, 2002).

It is not known how the key players in cell polarity are linked to the dynamic microtubule and actin cytoskeletons required for axis establishment and how the initially cortical Par protein localization is established and maintained. Furthermore, it remains unclear at which stages of development microtubule and actin dynamics are important to maintain polarity. In Drosophila, perturbing microtubules with drugs has no effect on Par protein localization, whereas disturbing the actin cytoskeleton affects Par-1 localization at mid-oogenesis (Doerflinger et al., 2006). This suggests a causal link between polarity proteins and the actin cytoskeleton in oocyte polarity maintenance.

Rho family GTPases are master regulators of the actin cytoskeleton (Jaffe and Hall, 2005). Among these is the small Rho-like GTPase Cdc42, which in addition to its role in regulating the actin cytoskeleton has been shown to interact biochemically and genetically with Par-6 (Betschinger et al., 2003; Georgiou et al., 2008; Harris and Tepass, 2008; Leibfried et al., 2008). Here, we report the central role of Cdc42 in oocyte establishment, Par protein regulation and maintenance of oocyte polarity.
MATERIALS AND METHODS

Fly stocks
The following fly stocks were used (see FlyBase for reference). Cdc42\(3\) bearing the lethal missense mutation G114A; Cdc42\(2\) bearing the lethal missense mutation G370A; Cdc42\(2\) bearing the viable missense mutation T311A; baz\(^{1106}\), a null allele; aPKC\(^{K06403}\), a strong hypomorphic allele; par-6\(^{DG26}\), a null allele; Arp3\(^{3F}\) bearing the lethal missense mutation C232A; and w\(^{1118}\).

Germline clones were recovered from flies of the following genotypes: (1) Cdc42\(3\) FRT19A/ubi\(\rightarrow\)nis-GFP hs\(\rightarrow\)Fип FRT19A, (2) Cdc42\(3\) FRT19A/ubi\(\rightarrow\)nis-GFP hs\(\rightarrow\)Fип FRT19A; mat\(\rightarrow\)ubi\(\rightarrow\)nis-RFP hs\(\rightarrow\)Fип FRT19A; (3) Cdc42\(2\) FRT19A/ubi\(\rightarrow\)nis-GFP hs\(\rightarrow\)Fип FRT19A, (4) baz\(^{1106}\) FRT9-2/ubi\(\rightarrow\)nis-GFP FRT9-2; hs\(\rightarrow\)Fип, (5) hs\(\rightarrow\)Fип; aPKC\(^{K06403}\) FRT42B/ubi\(\rightarrow\)nis-GFP FRT42B, (6) par-6\(^{DG26}\) FRT9-2/ubi\(\rightarrow\)nis-GFP hs\(\rightarrow\)Fип FRT9-2; Par-6\(^{Pro2B}\) (Hutterer et al., 2004) and (7) Cdc42\(3\) FRT19A/ubi\(\rightarrow\)nis-GFP hs\(\rightarrow\)Fип FRT19A/; UASp\(\rightarrow\)aPKC-CAAX/nanos\(\rightarrow\)Gal4\(\rightarrow\)VP16. Mutant clones were generated by heat shock of late L3 larvae for 1.5 hours at 37°C.

Ovaries were fixed in 4% PFA in 1:1 PBS:heptane for 20 minutes. For anti-Par-1 immunostaining, ovaries were allowed to recover for 24 hours at 18°C prior to dissection. For analysis of prolonged drug action, flies were bathed in DMSO (1:10 in water, control) or 1 mM Latrunculin A (Sigma) in yeast for 16 hours and subsequently dissected and processed for immunostaining as described above. The effectiveness of Latrunculin A was monitored by checking for defective nurse cell dumping and by measuring the average cell number per oocyte. Eggs were collected on apple juice plates and hatch rates scored by counting hatched and unhatched eggs after ageing for 24 hours at 25°C. Equal numbers of females and males were used to set up technical and biological replicates.

Drug treatment
Starved flies were fed with DMSO (1:10 in water, control) or 1 mM Latrunculin A (Sigma) in yeast for 16 hours and subsequently dissected and processed for immunostaining as described above. The effectiveness of Latrunculin A was monitored by checking for defective nurse cell dumping and by measuring the average number of oocytes per oocyte. Eggs were collected on apple juice plates and hatch rates scored by counting hatched and unhatched eggs after ageing for 24 hours at 25°C. Equal numbers of females and males were used to set up technical and biological replicates.

RESULTS
Loss of Cdc42 in the germline causes oogenesis arrest
It was previously reported that Cdc42 is required for polarity maintenance and egg chamber development in the Drosophila female germline (Genova et al., 2000). However, only hypomorphic EMS mutant alleles (Cdc42\(2\) and Cdc42\(2/1\) Cdc42\(2\)) were analyzed. We induced germline clones of the strong Cdc42\(3\) mutant in the Drosophila ovari. Loss of Cdc42 function led to arrest of oogenesis and mutant egg chambers developed only until stage 5 (Fig. 1A). Staining of nuclei with DAPI revealed that, from stage 4 onwards, mutant egg chambers contained 16 (normal) and 12 (mutant) nurse cells each in the normal 15 polyploid nurse cells and one oocyte (Fig. 1A; supplementary material Fig. S1A). Consistent with this, we observed that 4% of females developed stage 5 eggs, compared to 100% of wild-type females (Fig. 1A). Consistent with the arrest of oogenesis, we observed that 4% of females developed stage 5 eggs, compared to 100% of wild-type females (Fig. 1A).
Orb accumulates in one cell of young C(3)G were detected in just one of the 16 cells of cell of the cyst to enter meiosis (Takeo et al., 2011). Both Orb and complex marker C(3)G, which is specific to the oocyte, the only

specification. We visualized the cytoplasmic protein Orb, which could result from a loss of oocyte specification or a failure in its maintenance. To differentiate between these two possibilities, we performed immunostainings for early markers of oocyte

with the previous report, clones of the hypomorphic (weaker) Cdc42
to the anterior of just one cell in Cdc42 mutant egg chambers until stage 3 and was no longer observed at later stages (Fig. 1E). These results indicate that the oocyte is correctly specified in Cdc42 mutant germline clones, but that its fate is not maintained.

**Oocyte polarity maintenance is affected by loss of Cdc42**

Characteristic of the oocyte is the anterior-to-posterior migration of the centrosomes and Orb protein in region 2b of the gerarium. This translocation event was not observed in Cdc42 mutant cysts: Orb and CP309 remained localized at the anterior pole of the oocyte in region 3 and at stages 2-3 (Fig. 1E-G).

The failure to maintain oocyte polarity and the subsequent arrest of oogenesis in Cdc42 mutant egg chambers phenocopy loss of Par polarity proteins. Indeed, mutants for the anterolaterally localized Par-6, aPKC or Baz, as well as the posteriorly localized Par-1, also show a loss of oocyte polarity maintenance (Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b). We therefore tested whether polarized Par protein localization is affected by loss of Cdc42. As Par-6 and aPKC localization could not easily be detected in the young oocyte using available antibodies, we analyzed Baz localization in Cdc42 mutant egg chambers. Loss of Cdc42 resulted in loss of Baz from the anterolateral cortex of the oocyte (Fig. 1H).

Par-1 is present on the fusome in the gerarium and localizes at the posterior of the oocyte, where it counteracts Par-6/aPKC/Baz activity (Doerflinger et al., 2006; Huynh et al., 2001b; Shulman et al., 2000; Tomancak et al., 2000; Vaccari and Ephrussi, 2002). We tested the effect of loss of Cdc42 on Par-1 localization, taking two approaches. First, we performed immunostaining for Par-1, and observed that Par-1 localizes normally to the fusome in the gerarium in Cdc42 mutant clones (supplementary material Fig. S2A). Second, we expressed a GFP-Par-1 fusion protein specifically in the germline using the maternal tubulin promoter. However, as reported, this GFP-Par-1 did not display posterior localization in young egg chambers prior to stage 7 (Doerflinger et al., 2006). The localization of GFP-Par-1 was identical in wild-type and Cdc42 mutant egg chambers, although we observed weaker expression in the mutant than in wild-type egg chambers (supplementary material Fig. S2B). We conclude from the weaker expression pattern of the GFP fusion construct that, in young egg chambers, Par-1 localization or stability most likely also depends on Cdc42 function.

Cdc42 localizes to the germline and is needed for maintenance of oocyte polarity throughout oogenesis

In a previous report, ubiquitous expression of Myc-tagged Cdc42 in the fly and subsequent anti-Myc immunostaining revealed a cortical localization of Myc-Cdc42 in stage 10b germline cells: Myc-Cdc42 accumulated at the membrane and diffusely throughout the cytoplasm in nurse cells, but did not accumulate in the oocyte and was not detected at earlier stages (Genova et al., 2000). Using a specific anti-Cdc42 antibody (Harris and Teppas, 2008) we observed that, in the germline, Cdc42 localizes diffusely in the cytoplasm in the gerarium (Fig. 2A) and accumulates close to the anterolateral cortex of the young oocyte (Fig. 2B). As the signal is lost in Cdc42 mutant egg chambers, this pattern very likely represents the physiological localization of Cdc42 (Fig. 2C).
We furthermore tested for Cdc42 activity by expressing a FRET biosensor for Cdc42 in the *Drosophila* germline. This monomeric biosensor allows for FRET between YFP and CFP when GTP is bound to Cdc42 in the biosensor. Dominant-negative and dominant-active versions of the biosensor were used for OFF and ON measurements, respectively. Oocyte cortical regions of stage 4-5 egg chambers were used for the ratiometric analysis (YFP/CFP ratio). Efficiencies were significantly different between ON and OFF biosensors (Student’s t-test, \( P = 0.001 \)) and between OFF and wild-type biosensors (Student’s t-test, \( P = 0.024 \)). This analysis revealed that Cdc42 is present in its active conformation in the germline (Fig. 2D,E).

Cdc42 is maternally required for correct embryogenesis (Genova et al., 2000; Kamiyama and Chiba, 2009). We assayed the hatching of larvae lacking wild-type maternal or both maternal and zygotic Cdc42. We analyzed the hypomorphic allele *Cdc42*\(^{2} \) (Genova et al., 2000), which is a viable mutant allele. The hatching rate of the progeny resulting from *Cdc42*\(^{2} \) mutant females crossed to *Cdc42*\(^{2} \) males was reduced compared with wild type (Fig. 3A) and remained low when *Cdc42*\(^{2} \) mutant females were crossed to wild-type males. Thus, Cdc42 is maternally required for normal embryogenesis.

The hatching defect suggested that, in addition to its early requirement, Cdc42 might be needed at later stages of oogenesis. Since stage 9/10 homozygous mutant egg chambers could not be obtained using the *Cdc42*\(^{2} \) mutant allele, we used the weaker *Cdc42*\(^{2} \) allele for analysis in mid-oogenesis. *Cdc42*\(^{2} \) germline clones displayed a mislocalized nucleus and mislocalized Staufen protein (25% mislocalization; supplementary material Fig. S3A,B). We also observed a lack of posterior accumulation of Vasa in 30% of stage 9 *Cdc42*\(^{2} \) mutant clones, which is marked by loss of nuclear GFP. Asterisks mark oocytes of interest and arrows indicate specific protein localization. (D,E) FRET measurements show that Cdc42 is present in its active conformation in the germline. A membrane-targeted FRET biosensor for Cdc42 was expressed in the germline with the driver *oskar-Gal4* (D; \( n = 20 \)). Dominant-negative Cdc42N17 \( (n=19) \) biosensor and dominant-active Cdc42V12 \( (n=16) \) biosensor were used to measure the FRET efficiency rates for the ON and OFF state, respectively (E). \( *P = 0.024, ***P = 0.001 \). Standard error (s.e.) is depicted. Scale bars: 50 \( \mu \)m in A; 25 \( \mu \)m in B-D.

**Cdc42 and the Par complex interact to maintain oocyte polarity**

Cdc42 is an upstream activator of the Par-6/aPKC/Baz complex in mammalian and *Drosophila* epithelial cells (Hutterer et al., 2004; Joberty et al., 2000). Since the early oocyte polarity defects observed in *Cdc42*\(^{2} \) mutant germline clones resemble those found in par-6, aPKC or baz mutants, we reasoned that they might be due to loss of the Par complex from the anterior of the oocyte (Fig. 1H; supplementary material Fig. S2B). We addressed the possible mechanistic link between Cdc42 and the Par complex in oocyte polarity maintenance by performing genetic and biochemical interaction assays. Reducing the gene copy number of both *Cdc42* and *aPKC* led to loss of oocyte polarity in 13.6% of the stage 10 egg chambers (Table 1, Fig. 4A). Furthermore, expression of Par-6ΔP, which cannot bind to Cdc42 (Hutterer et al., 2004), in a
Fig. 3. Cdc42 is needed for oocyte polarity maintenance throughout oogenesis. (A) The hatching rate of eggs laid by Cdc42 mutants is reduced, regardless of homozygous or heterozygous mutant zygotic state (mutant or wild-type paternal Cdc42 contribution). Error bars represent ±s.e. Significance was calculated using Student’s t-test, with *P* ≤ 0.05 for w1118 × w1118 versus w1118 × Cdc422; *P* ≤ 0.001 for w1118 × w1118 versus Cdc422 × Cdc422; and *P* ≤ 0.179 for Cdc422 × Cdc422 versus Cdc422 × w1118; n.s., not significant. (B-D) Expression of shRNA against Cdc42 leads to downregulation of Cdc42 in the germline. Overall Cdc42 protein levels (as detected by western blot) are strongly reduced in shRNA-expressing ovaries (B). Khc provides a loading control. Cdc42 is present at the anterolateral cortex of control egg chambers (C), but lost in shRNA-expressing egg chambers (D). Asterisks mark oocyte. (E-H) Anterolateral localized Baz (E) is reduced in shRNA-expressing egg chambers (F) and posterior localization of Par-1 (G) is lost in shRNA-expressing egg chambers (H). Arrows indicate protein localization. (I,J,O) Expression of Cdc42 shRNA results in loss of osk mRNA from the posterior pole of stage 9/10 egg chambers. osk localizes normally to the posterior of the egg chamber in the control (I), but not in shRNA-expressing egg chambers (J). The effect is stronger at stage 9 than at stage 10 (O; n=102 for shRNA expression at stage 9; n=87 for control at stage 9; n=122 for shRNA expression at stage 10; n=144 for control at stage 10). (K,L,P) Expression of Cdc42 shRNA results in reduced posterior localization of Osk protein. Osk localizes normally to the posterior of the egg chamber in the control (K), but not in shRNA-expressing egg chambers (L). (P) n=36 for shRNA expression at stage 9; n=28 for control at stage 9; n=49 for shRNA expression at stage 10; n=35 for control at stage 10. (M,N,Q) Expression of Cdc42 shRNA results in reduced levels of grk mRNA at the anterodorsal corner of stage 9/10 oocytes. grk localizes normally to the anterodorsal corner of the oocyte in the control (M), but is weaker in shRNA-expressing egg chambers (N). The effect is stronger at stage 10 than at stage 9 (Q; n=71 for shRNA expression at stage 9; n=79 for control at stage 9; n=134 for shRNA expression at stage 10; n=100 for control at stage 10). (R) Downregulation of Cdc42 leads to defects in nurse cell dumping, resulting in an enlarged nurse cell compartment. Inset shows a wild-type late-stage egg chamber. Scale bars: 25 µm.
par-6^{H11032} mutant background also led to loss of oocyte polarity (Fig. 4B). Finally, using anti-Cdc42 antibody we were able to co-immunoprecipitate aPKC from ovarian extracts, confirming the Par-Cdc42 interaction in vivo. This shows that Cdc42, Par-6 and aPKC interact and that the phenotype observed in Cdc42^{3/+} mutant egg chambers is most likely due to the loss of Par/Cdc42 complex interaction (Fig. 4C).

Binding of Par-6 to aPKC and Cdc42 causes a conformational change in Cdc42-GTP (Garrard et al., 2003) and leads to aPKC activation in Drosophila epithelial cells (Hutterer et al., 2004). Hence, some of the defects that occur when expressing a dominant-negative form of Cdc42 in the Drosophila embryonic neuroectoderm could be rescued by the concomitant overexpression of membrane-tethered active aPKC (Harris and Teppas, 2008), and membrane-tethered aPKC has been shown to recruit polarity proteins to the membrane (Sotillos et al., 2004). We attempted to rescue the Cdc42^{3/+} mutant phenotype by expression of the membrane-tethered aPKC in the germline using the germline-suitable Gal4/UASp system. This was not able to rescue the arrest of oogenesis at stage 5 (Fig. 4D) and few mutant clones were obtained. Furthermore, expression of membrane-tethered aPKC in a wild-type background resulted in mislocalization of the endogenous polarity proteins and Cdc42, which showed no specific enrichment at the membrane despite the enriched signal of aPKC at this site (supplementary material Fig. S6A-E). The overexpression also eventually led to arrest of oogenesis. This indicates that the correct localization of the endogenous proteins is required for oocyte polarity and that Par complex function is spatially very tightly regulated in the germline.

A feedback loop between Par proteins and Cdc42 ensures a proper actin cytoskeleton and polarity maintenance

Cdc42 is known to act as an upstream regulator of the actin cytoskeleton in Drosophila by activation of its effectors Cip4, WASp and Arp2/3 (Georgiou et al., 2008; Leibfried et al., 2008). The Drosophila oocyte shows a strong actin cytoskeleton at the anterolateral cortex from stage 4 onwards, which until now has not been linked to Par-6/aPKC/Baz. Females expressing the shRNA against Cdc42 did not lay any eggs and late-stage egg chambers displayed an enlarged nurse cell compartment typical of a failure in nurse cell dumping (Fig. 3R), which is an actin-dependent process (Cooley and Theurkauf, 1994). Further investigation revealed that actin was less abundant in Cdc42^{3/+} mutant egg chambers and specifically disrupted at the anterolateral cortex in Cdc42^{3/+} mutant oocytes (Fig. 4E,E; supplementary material Fig. S7). The actin-rich ring canals were not affected by loss of Cdc42 (Fig. 4E).

To test the role of the actin cytoskeleton in early polarity maintenance, we fed flies with Latrunculin A. Orb protein was localized in the oocyte under these conditions, but its typical anteroposterior relocation did not occur, indicating loss of oocyte polarity (Fig. 4F,G). We did not observe a 16-nurse-cell phenotype after Latrunculin A treatment. This could be explained by the observed failure of endoreplication (not shown), which is required for oocyte-to-nurse cell fate change. Alternatively, the 16-nurse-cell phenotype observed in Cdc42/Par mutants might be due to failure of a distinct, actin-independent, mechanism. The microtubule network, which has been shown to be required for oocyte specification (Koch and Spitzer, 1983; Theurkauf et al., 1993), was not visibly affected by Latrunculin A treatment (supplementary material Fig. S8A-C, Movies 1-3). Although Cdc42 is not the sole actin regulator, technical reasons prevented genetic analysis of other small GTPases, such as Rho and Rac, in early oogenesis. Experiments using drugs to specifically inhibit Rho, Rac or Cdc42 were inconclusive (supplementary material Table S1).

These data place Cdc42/Par-6/aPKC/Baz upstream of an actin-mediated process in oocyte polarity establishment. Reducing the gene copy number of either of Cdc42, aPKC or baz in combination with Arp3 led to mislocalization of Orb to the anterior in 11.5-17.5% of the young egg chambers analyzed (Table 1, Fig. 4H-J). As Cdc42 is the sole known actin regulator of the Cdc42/Par-6/aPKC/Baz complex, and loss of function of Cdc42 or the Par proteins leads to defects that can be phenocopied by disrupting the actin cytoskeleton, we speculated that the genetic Cdc42−Par-6/aPKC/Baz interaction might be reciprocal. Therefore, we analyzed the distribution of the actin cytoskeleton and Cdc42 localization in baz^{H106} null mutant egg chambers and in hypomorphic aPKC^{H06403} mutant egg chambers. Both the anterolateral enrichment of actin and Cdc42 localization were lost in baz^{H106} and aPKC^{H06403} egg chambers (Fig. 4K,L; supplementary material Fig. S9A-C) indicating that, although Cdc42 is required for correct Par complex localization, the Par complex in turn is needed for localized Cdc42 accumulation and thus for maintenance of the actin cytoskeleton. In line with this conclusion, Baz remains localized after disruption of the actin cytoskeleton by Latrunculin A (Fig. 4M). Thus, Par/Cdc42-mediated regulation of the actin cytoskeleton at the anterolateral cortex of the oocyte is crucial for the maintenance of oocyte polarity at early stages (Fig. 4N).

DISCUSSION

Our findings show that Cdc42 is required for oocyte polarity throughout oogenesis. We have found that: (1) Cdc42 localizes to the anterolateral cortex of the young oocyte; (2) Cdc42 interacts with Par proteins in the germline in vivo; (3) mutants for Cdc42, aPKC or Baz display a disrupted actin cytoskeleton at the anterolateral cortex; and (4) disrupting the actin cytoskeleton with Latrunculin A results in loss of anterior-to-posterior movement of

| Table 1. Genetic interaction assays between Cdc42 and aPKC and between Cdc42/aPKC/Baz and Arp3 |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Normal | 116 | 163 | 140 | 116 | 78 | 146 | 118 | 97 |
| Mislocalized at anterior | 3 | 4 | 21 | 5 | 1 | 19 | 25 | 20 |
| Absent | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 |
| Total | 119 | 167 | 162 | 121 | 79 | 165 | 143 | 117 |
| Orb mislocalization (%) | 2.5 | 2.4 | 13.6 | 4.1 | 1.3 | 11.5 | 17.5 | 17.1 |

Cdc42 and aPKC interact genetically, as do Cdc42/aPKC/Baz and Arp3.
the oocyte-specific protein Orb, phenocopying loss of Cdc42 or the Par proteins. Thus, the cortical actin cytoskeleton is crucial for the establishment of oocyte polarity.

This is in line with previous observations linking the actin cytoskeleton and Par proteins in the generation of cell polarity. Loss of Baz results in an increase in actin protrusions in *Drosophila* epithelia (Georgiou and Baum, 2010) and a decrease in actin at synapses (Ramachandran et al., 2009). In *C. elegans*, active CDC-42 localizes to the anterior during the polarity maintenance phase (Kumfer et al., 2010), when it is important for PAR-6 localization (Motegi and Sugimoto, 2006), and the anterior actin cap is depleted in par-3 mutants during polarity establishment (Kirby et al., 1990). Similar to our observations, actin depolymerization does not affect Par protein localization in *C. elegans* (Goehring et al., 2011). By contrast, drug-induced actin depolymerization has been shown to disrupt Baz apical localization during cellularization (Harris and Peifer, 2005) and to interfere with its cortical association during gastrulation in *Drosophila* (Simões et al., 2010).

Fig. 4. See next page for legend.
Fig. 4. Cdc42 and Par proteins cooperate to organize the actin cytoskeleton and establish oocyte polarity. (A) Cdc42<sup>−/−</sup> heterozygous or aPKC<sup>k06403</sup> heterozygous oocytes do not lose polarity, whereas transheterozygous oocytes display anteriorly mislocalized Orb protein. Inset is an enlargement of an egg chamber. (B) par-6 mutant egg chambers expressing Par-6<sup>AP</sup>, a Par-6 that cannot bind to Cdc42, lose specific Orb localization at the posterior of the oocyte (top; 67%, n=21, stage 2-4), leading to arrest of oogenesis (bottom). (C) Co-immunoprecipitation of aPKC and Cdc42. Ovarian extracts were incubated with anti-Cdc42 or anti-GFP (control). Note that the secondary antibody used to detect anti-Cdc42 antibody cross-reacts with IgG light chain in both the specific and the control pull-down. (D) Expression of membrane-tethered aPKC in Cdc42<sup>−/−</sup> mutant egg chambers does not rescue oocyte polarity (83%, n=6). Mutant clones are marked by loss of nuclear GFP, the GFP channel is shown alone in the top panel. (E) Cdc42<sup>−/−</sup> mutant egg chambers marked by loss of nuclear GFP lack a pronounced actin cytoskeleton at the anterolateral cortex of the oocyte (85%, n=13). Note that the ring canals remain intact in the mutant (double arrowheads). The phalloidin channel is shown alone in the bottom panel. (F) Quantification of Rhodamine-phalloidin staining in wild-type and Cdc42<sup>−/−</sup> mutant egg chambers (germline), oocytes (middle bars), and the anterior and lateral regions of oocytes (oocyte anterolateral; avoiding the posterior, which is in contact with the somatic cells). Error bars indicate s.e. See supplementary material Fig. S7 for an indication of the regions chosen for analysis. (G) Disrupting the actin cytoskeleton with Latrunculin A phenocopies loss of Cdc42 or Par proteins. Orb localizes normally in control egg chambers (F; 90%, n=50), whereas it is mislocalized in Latrunculin A-treated egg chambers (G; 37%, n=81). (H-J) Cdc42<sup>−/−</sup>, aPKC<sup>k06403</sup>, baz<sup>7106</sup> or Ars3<sup>588</sup> heterozygous oocytes do not lose polarity, whereas transheterozygous oocytes display anteriorly mislocalized Orb protein. (K) baz<sup>7106</sup> mutant egg chambers marked by loss of nuclear GFP result in loss of the pronounced actin cytoskeleton at the anterolateral cortex of the oocyte (50%, n=4). The actin cytoskeleton is marked by fluorescently labeled phalloidin. (L) baz<sup>7106</sup> mutant egg chambers (marked by loss of nuclear GFP) exhibit absence of Cdc42 from the anterolateral cortex of the oocyte (100%, n=5). (M) Baz localization is not affected by Latrunculin A treatment. (N) Proposed mechanism of oocyte polarity regulation. A quaternary complex comprising Cdc42, Par-6, aPKC and Baz is needed at the anterior of the oocyte for localized branched actin formation. This allows for the polymerization of the actin, reflected by the translocation of Orb protein. Asterisks mark oocytes of interest; arrowheads pointing to the right mark anterior protein localization; arrowheads pointing to the left mark posterior protein localization. Yellow arrowheads (J) mark anterior protein localization. Arrows point to specific actin (E,K), Cdc42 (L) and Baz> GFP (M) enrichment. Scale bars: 25 μm.

Although the molecular relationship between Par proteins and actin has not been clearly delineated, in mammals Par-3 (Pard3) associates with actin regulators, including the RacGEF Tiam1 and LIM kinase 2 (Chen and Macara, 2005; Chen and Macara, 2006; Mertens et al., 2005; Nishimura et al., 2005; Zhang and Macara, 2006). In our study, we have shown that Cdc42 localization depends on the Par complex and that Cdc42, aPKC, Baz and Par-6 interact in vivo in biochemical or genetic assays. This interaction is required for oocyte polarity. Par-6 interacts biochemically with Cdc42 and Baz via its semi-CRIB and PDZ domains and via its PB1 domain with the PB1 domain of aPKC. Baz interacts biochemically with the kinase domain of aPKC. Indeed, a quaternary complex of Myc-Cdc42, HA-Par-6b, PKC<sup>τ</sup>-A and Par-3 can be isolated from transfected COS-7 cells (Joberty et al., 2000). In Drosophila, a Baz mutant lacking its aPKC-interaction domain supports early oogenesis and an aPKC mutant that cannot bind Par-6 also develops late egg chambers (Doerflinger et al., 2010; Kim et al., 2009). Together, these results and our data indicate that interaction of Cdc42, Par-6, aPKC and Baz is required for their correct function in the germline, and that the binding of aPKC to either Par-6 or to Baz is sufficient to ensure this interaction, highlighting the role of all three Par proteins in actin regulation via their interaction with Cdc42. This quaternary relationship seems important for the regulation of polarity establishment, whereas studies in mature epithelial cells have delineated separate functions of Par-6/aPKC/Cdc42 and Baz for polarity maintenance (Morais-de-Sá et al., 2010; Georgiou et al., 2008; Harris and Teopoulos, 2008; Leibfried et al., 2008).

Early oocyte polarity and its maintenance were previously linked to the microtubule network. Microtubules play an important role in early oogenesis, as their disruption with Colchicine leads to a 16-nurse-cell phenotype (Koch and Spitzer, 1983; Theurkauf et al., 1993). Indeed, oocyte specification depends on the accumulation of the oocyte-specific protein BicD, which is a component of the microtubule-related dynactin complex (Mach and Lehmann, 1997; Theurkauf et al., 1993; Huynh and St Johnston, 2000).

Our results point to a sequential involvement of actin and microtubules in polarizing the oocyte: in the early stages, after oocyte specification, the Par proteins together with Cdc42 establish cortical domains and a pronounced cortical actin cytoskeleton. The interdependence of these proteins for their localization persists during oogenesis, allowing for robustness of symmetry breaking. At later stages, knockdown of Cdc42 results in reduced amounts of Baz and Par-1 at the anterior and posterior of the oocyte, respectively. As Par-1 is required for microtubule organization (Becalska and Gavis, 2009; Benton and St Johnston, 2002; Cox et al., 2001a), this most likely leads to the observed mislocalization of axis determinants. Similarly, disrupting the actin cytoskeleton with drugs or by knockdown of actin-binding proteins has been shown to result in bundling of microtubules and premature ooplasmic streaming, leading to loss of oocyte polarity (Doerflinger et al., 2006; Manseau et al., 1996). Hence, microtubules act in oocyte specification and late polarity events, whereas Cdc42 and actin dominate in the establishment and maintenance of polarity in the developing oocyte.

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