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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.

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MATERIALS AND METHODS

Fly stocks

The following fly stocks were used (see FlyBase for reference). *Cdc42*^{3} bearing the lethal missense mutation G114A; *Cdc42*^{4} bearing the lethal missense mutation G370A; *Cdc42*^{2} bearing the viable missense mutation T311A; *baz*^{xi106}, a null allele; *aPKC*^{K06403}, a strong hypomorphic allele; *par-6*$^{\Delta 226}$, a null allele; *Arp3*^{83F} bearing the lethal missense mutation C232A; and *w*^{1118}.

Germline clones were recovered from flies of the following genotypes: (1) *Cdc42*^{3} *FRT19A/ubi>nls-GFP hs>Flp FRT19A*, (2) *Cdc42*^{3} *FRT19A/ubi>nls-RFP hs>Flp FRT19A;; matTub>GFP-Par-1 N1S* (Huynh et al., 2001b), (3) *Cdc42*^{4} *FRT19A/ubi>nls-GFP hs>Flp FRT19A*, (4) *baz*^{xi106} *FRT9-2/ubi>nls-GFP FRT9-2; hs>Flp*, (5) *hs>Flp*; *aPKC*^{K06403} *FRT42B/ubi>nls-GFP FRT42B*, (6) *par-6*$^{\Delta 226}$ *FRT9-2/ubi>nls-GFP hs>Flp FRT9-2; Par-6Pro2B* (Hutterer et al., 2004) and (7) *Cdc42*^{3} *FRT19A/ubi>nls-GFP hs>Flp FRT19A;; UASp>aPKC-CAAX/nanos>Gal4VP16*. Mutant clones were generated by heat shock of late L3 larvae for 1.5 hours at 37°C.

Ovaries of the following genotypes were analyzed: (1) *oskar>Gal4/+* (Telley et al., 2012), (2) *oskar>Gal4/UASp>shRNA-Cdc42*, (3) UAS>Par-1 N1S; *oskar>Gal4/UASp>shRNA-Cdc42*, (4) *Cdc42*^{3}*, FRT19A/+*, (5) *aPKC*^{K06403} *FRT42B/+*, (6) *Cdc42*^{3} *FRT19A/+; aPKC*^{K06403}*FRT42B/+*, (7) *Arp3*^{83F}*/+*, (8) *Cdc42*^{3} *FRT19A/+;; Arp3*^{83F}*/+*, (9) *baz*^{xi106} *FRT9-2/+;; Arp3*^{83F}*/+*, (10) *aPKC*^{K06403} *FRT42B/+; Arp3*^{83F}*/+*, (11) *nanos>Gal4*/UAS>aPKC-CAAX, (12) *nanos>Gal4/+*, (13) *w*^{1118}, (14) *baz*^{CC01941} (a Baz trap line), (15) *Cdc42*^{4} *FRT19A/ubi>nls-GFP hs>Flp FRT19A*, (16) *Cdc42*^{4} *FRT19A/ovoD hs>Flp FRT19A; matTub>GFP:Staufen*, (17) *FRT19A/ovoD hs>Flp FRT19A; matTub>GFP:Staufen*, (18) *FRT19A/ovoD hs>Flp FRT19A*, (19) Jupiter^{3G00147} and (20) *oskar>Gal4*/UASp-EGFP.

shRNA and UASp>aPKC-CAAX constructs

An shRNA construct targeting the *Cdc42* sequence 5′-AGGAAGTGCAAATTCTTATAA-3′ was cloned into pValium22 (Ni et al., 2008). For analysis of shRNA-mediated effects, freshly eclosed females of knockdown and control genotype were fed on yeast for 1 day at 29°C prior to dissection. aPKC-CAAX vector was obtained from Sonsoles Campuzano (CBM, Madrid, Spain) and subcloned into pUASp vector using *Not*I and *Xba*I restriction sites.

Förster resonance energy transfer (FRET) biosensor

For the wild-type FRET biosensor, Raichu/1069x (T. Nakamura and M. Matsuda, University of Osaka, Japan) was cloned into a UASp vector suitable for ΦC31 integrase-mediated transformation. For OFF and ON biosensors, N17 and V12 point mutations, respectively, were introduced with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Transformants were crossed to the *oskar>Gal4* driver line. Analysis was performed using the ratiometric method, calculating the ratio of YFP/CFP at the oocyte cortex. Student's *t*-test was used for statistical analysis.

Immunohistochemistry

Ovaries were dissected in PBS and processed for immunostaining as described (Vanzo and Ephrussi, 2002) using the following antibodies: rabbit anti-GFP (1:2000, Invitrogen), mouse anti-Orb [1:250, 6H8 and 4H8 from the Developmental Studies Hybridoma Bank (DSHB)], rat anti-Vasa (1:1000, DSHB), mouse anti-C(3)G (1:500, gift of Scott Hawley, Stowers Institute, Kansas City, USA), rabbit anti-CP309 (1:500, gift of Yixian Zheng, Carnegie Institution, Baltimore, USA), rabbit anti-Baz (1:1000) and rat anti-Par-6 (1:500) (gifts of Andreas Wodarz, University of Göttingen, Germany), guinea-pig anti-Par-1 (1:50, gift of Jocelyn McDonald, Lerner Institute, Cleveland, USA), guinea-pig anti-Cdc42 (1:1000, gift of Ulrich Tepass, University of Toronto, Canada), rabbit anti-Osk [1:3000 (Vanzo and Ephrussi, 2002)] and rabbit anti-aPKC (1:1000, Sigma). For anti-Cdc42 immunostaining, ovaries were dissected in PEMS buffer (0.1 M Pipes, 2 µM $MgSO_4$, 50 µM EGTA) and fixed with 6% paraformaldehyde (PFA) for 20 minutes in PEMS buffer. For anti-Par-1 immunostaining, ovaries were fixed in 4% PFA in 1:1 PBS:heptane for 20 minutes. For staining of the actin cytoskeleton, Alexa Fluor 568 phalloidin (Invitrogen) was used at 1:100 on ovaries dissected in PEMS buffer. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). For microtubule stainings, ovaries were dehydrated with methanol after PFA fixation and kept overnight at –20°C. Ovaries were subsequently rehydrated and immunostaining was performed as described using FITC-conjugated mouse anti-tubulin (Abcam).

Fluorescent *in situ* hybridization coupled with immunodetection was performed using a digoxigenin-labeled *osk* 3′UTR or *gurken* (*grk*) antisense probe (at a final concentration of 0.5 ng/µl).

Confocal images were taken using oil-immersion 40× and 60× objectives on a Zeiss LSM 510 or 780 confocal microscope and visualized using Zeiss Confocal Software.

Western blotting

The following antibodies were used for western blot analysis: guinea-pig anti-Cdc42 (1:2000, gift of Ulrich Tepass, University of Toronto, Canada), rabbit anti-Khc (1:20,000, Cytoskeleton), rabbit anti-Baz (1:4000, gift of Andreas Wodarz), rabbit anti-aPKC (1:2000, Santa Cruz), HRP-conjugated anti-rabbit IgG (1:5000, GE Healthcare) and POD-conjugated anti-guinea-pig IgG (1:5000, Sigma). Quantification was performed using ImageJ (NIH).

Immunoprecipitation

Co-immunoprecipitation was performed on dissected *w*^{1118} ovaries. Lysate [buffer comprised 50 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl, 0.5% NP40, Complete Protease Inhibitor Cocktail (Roche)] was precleared on Dynabeads (Life Technologies). Rabbit anti-GFP (control; Invitrogen) and guinea-pig anti-Cdc42 (gift of Ulrich Tepass) were bound to Protein A Dynabeads and subsequently incubated with the precleared protein lysate for 1 hour at 25°C. Bound aPKC protein was analyzed on a western blot. Precleared lysate was used as input control.

Maternal-effect analysis

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 the crosses.

Drug treatment

Starved *w*^{1118} 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 mean intensity of Rhodamine-phalloidin (Invitrogen), which was reduced by 50% upon Latrunculin A treatment [mean intensity DMSO, 23±1.22 (standard error); mean intensity Latrunculin A, 12±1.15; n=10). For analysis of prolonged drug action, flies were allowed to recover for 24 hours at 18°C prior to dissection. For Rho GTPase drug experiments, ovaries from *w*^{1118} females were bathed in Schneider's medium supplemented with 15% fetal calf serum, 1% penicillin-streptomycin, 2 mg/ml insulin and the drugs at indicated concentrations at 25°C. After 4 hours of incubation, ovaries were fixed with 4% PFA in PBS and immunostained as described.

RESULTS

Loss of Cdc42 in the germline causes oogenesis arrest

It was previously reported that Cdc42 is not required for polarity maintenance and egg chamber development in the *Drosophila* female germline (Genova et al., 2000). However, only hypomorphic EMS mutant alleles (*Cdc42*^{4} and *Cdc42*^{4}/*Cdc42*^{6}) were analyzed. We induced germline clones of the strong *Cdc42*^{3} mutant in the *Drosophila* ovary. 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 polyploid nurse cells instead of the normal 15 polyploid nurse cells and one oocyte (Fig. 1A; supplementary material Fig. S1A). Consistent

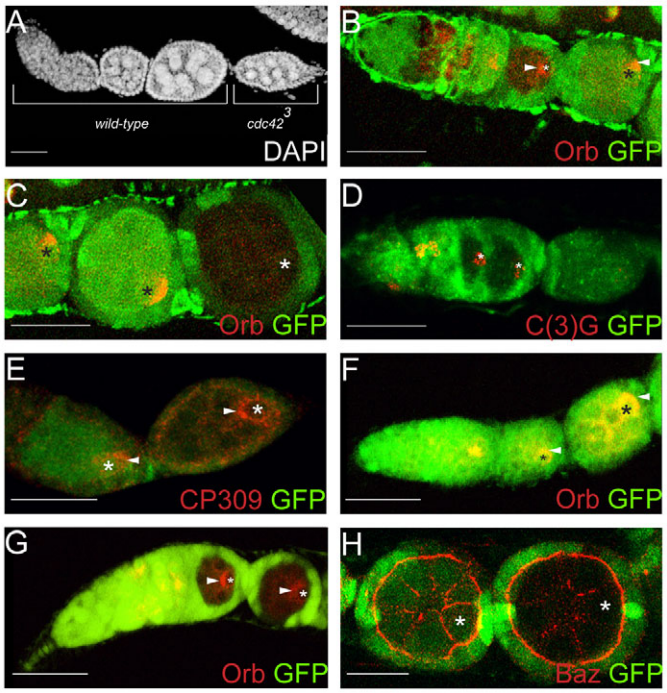

Fig. 1. Loss of Cdc42 leads to arrest of oogenesis and a failure to establish oocyte polarity. (**A**) *Cdc42*^{3} mutant egg chambers develop only until stage 5 and display 16 nurse cells. (B-H) *Cdc42*^{3} mutant clones marked by loss of nuclear GFP. (**B**,**C**) The oocyte-specific protein Orb accumulates in one cell of young *Cdc42*^{3} mutant egg chambers (B), but is lost at later stages (C) (100%, *n*=24). (**D**) The meiosis marker C(3)G accumulates in one cell of young *Cdc42*^{3} mutant egg chambers, but is lost at later stages (100%, *n*=16). (**E**) *Cdc42*^{3} germline clones display the centrosomal protein CP309 in the oocyte, but the usual anteroposterior translocation does not take place (100%, *n*=13). (**F**,**G**) The oocyte-specific protein Orb is found at the posterior of the oocyte in young wild-type egg chambers (F), but does not translocate from the anterior to the posterior in *Cdc42*^{3} mutant egg chambers (G; 78%, *n*=9). (**H**) The Par complex protein Baz is lost from the anterolateral cortex in *Cdc42*^{3} mutant egg chambers (89%, *n*=18). Asterisks mark oocytes of interest; arrowheads pointing to the right mark anterior protein localization; arrowheads pointing to the left mark posterior protein localization. Scale bars: 50 μm.

with the previous report, clones of the hypomorphic (weaker) *Cdc42*^{4} allele displayed the same, albeit less penetrant, phenotype (supplementary material Fig. S1B).

The oogenesis default observed in *Cdc42* mutant egg chambers 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 specification. We visualized the cytoplasmic protein Orb, which accumulates first in the two pro-oocytes and later becomes restricted to the oocyte in a microtubule-dependent manner (Lantz et al., 1994; Mach and Lehmann, 1997; Suter et al., 1989; Wharton and Struhl, 1989). We examined the presence of the synaptonemal complex marker C(3)G, which is specific to the oocyte, the only cell of the cyst to enter meiosis (Takeo et al., 2011). Both Orb and C(3)G were detected in just one of the 16 cells of *Cdc42*^{3} mutant egg chambers in region 3 in the germarium and at stages 2 and 3, but no staining was observed in later stages (Fig. 1B-D). In early oogenesis, centrosomes migrate into the oocyte (Bolívar et al., 2001; Grieder et al., 2000; Kawaguchi and Zheng, 2004). Consistent with the presence of Orb and C(3)G in young *Cdc42*^{3} mutant egg chambers, the centrosomal protein CP309 was localized to the anterior of just one cell in *Cdc42*^{3} 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*^{3} 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 germarium. This translocation event was not observed in *Cdc42*^{3} 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*^{3} 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*^{3} 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 germarium 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 germarium in *Cdc42*^{3} 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*^{3} 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 Tepass, 2008) we observed that, in the germline, Cdc42 localizes diffusely in the cytoplasm in the germarium (Fig. 2A) and accumulates close to the anterolateral cortex of the young oocyte (Fig. 2B). As the signal is lost in *Cdc42*^{3} mutant egg chambers, this pattern very likely represents the physiological localization of Cdc42 (Fig. 2C).

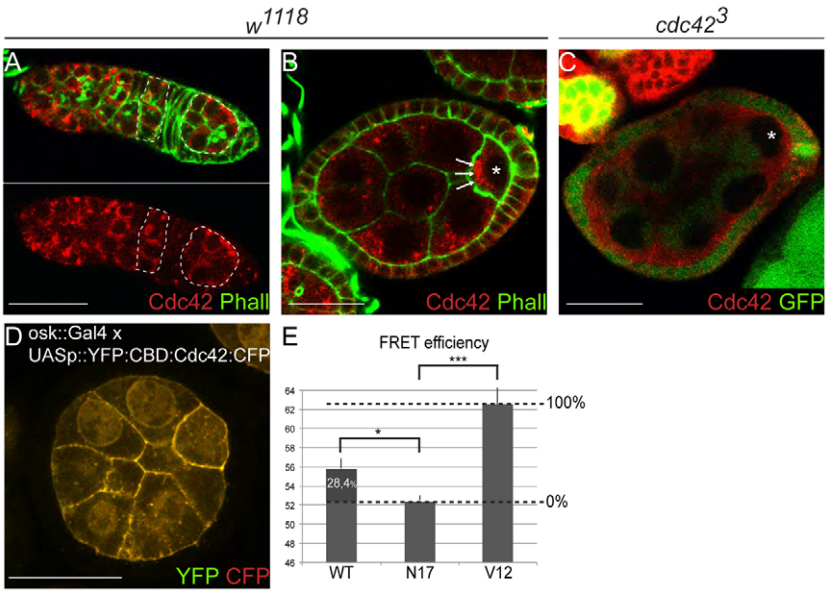

Fig. 2. Cdc42 localizes to the anterolateral cortex of the oocyte. (**A**) Cdc42 localization in the germarium of w^{1118} ovaries. The actin cytoskeleton is marked by fluorescently labeled phalloidin. Dashed lines encircle young cysts in the germarium. (**B**) Cdc42 localizes to the anterolateral cortex of the young oocyte in w^{1118} ovaries. (**C**) Cdc42 staining is lost in $Cdc42^3$ mutant clones, which are 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 μm in A; 25 μm in B-D.

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 $Cdc42^3$ mutant allele, we used the weaker $Cdc42^4$ allele for analysis in mid-oogenesis. $Cdc42^4$ 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 the egg chambers analyzed (supplementary material Fig. S3C,D), a defect not reported previously (Genova et al., 2000).

To further confirm that Cdc42 is also required at later stages, we constructed a miR-1 scaffold-based (Ni et al., 2008) small hairpin RNA (shRNA) against *Cdc42* that we expressed in the germline using the strong maternal driver *oskar-Gal4* (supplementary material Fig. S4). Expression of this construct led to an 80% reduction in Cdc42 protein levels on western blots (Fig. 3B) and loss of detectable Cdc42 signal from the anterolateral cortex of the oocyte (Fig. 3C,D). The early oocyte polarity marker Orb mislocalized to the anterior of the oocyte in 25% of young egg chambers analyzed (supplementary material Fig. S5), indicating that the knockdown phenocopies loss of Cdc42.

We then monitored the effect of Cdc42 downregulation on oocyte polarity at later stages. We analyzed well-characterized oocyte polarity markers: anterolaterally localized Baz, posteriorly localized *osk* mRNA and Par-1, and dorsoventrally localized *grk* mRNA (Benton and St Johnston, 2003; Ephrussi et al., 1991; Kim-Ha et al., 1991; Neuman-Silberberg and Schüpbach, 1993; Shulman et al., 2000). Cdc42 downregulation by shRNA led to a reduction of anterolateral localization of Baz, indicating that Cdc42 is required at later stages of oogenesis for correct localization of the Par complex (Fig. 3E,F). Similarly, localization of GFP-Par-1 to the posterior was abolished in mid-oogenesis when Cdc42 protein levels were reduced (Fig. 3G,H). Expression of shRNA against *Cdc42* also led to very weak polar localization or absence of *osk* mRNA at the posterior in 87% of stage 9-10 egg chambers (Fig. 3I,J,O) and, consequently, normal Osk protein localization was only detected in 4% and 6% of stage 9 and stage 10 egg chambers, respectively (Fig. 3K,L,P). Reduction of Cdc42 also affected *grk* localization and nuclear position (13% of the stage 10 egg chambers showed mislocalization; Fig. 3M,N,Q). We conclude that Cdc42 is required for oocyte polarity maintenance at later stages of oogenesis.

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 $Cdc42^3$ 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 young egg chambers analyzed (Table 1, Fig. 4A). Furthermore, expression of Par-6ΔP, which cannot bind to Cdc42 (Hutterer et al., 2004), in a

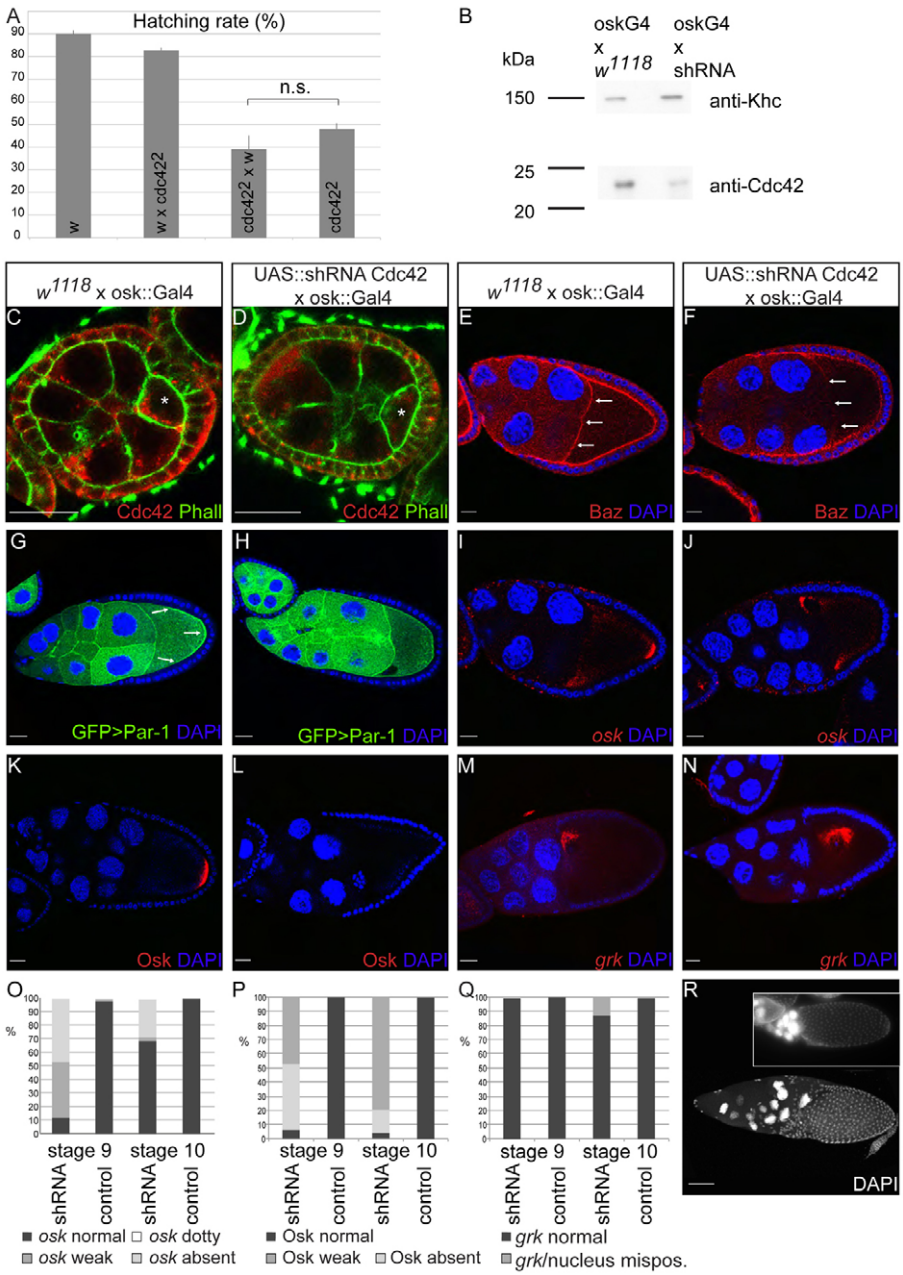

Fig. 3. Cdc42 is needed for oocyte polarity maintenance throughout oogenesis. (**A**) The hatching rate of eggs laid by *Cdc42*^{2} mutant flies is reduced, regardless of homozygous or heterozygous mutant zygotic state (mutant or wild-type paternal Cdc42 contribution). Error bars represent s.e. Significance was calculated using Student's *t*-test, with *P*=0 for $w^{1118} \times w^{1118}$ versus *Cdc42*$^{2} \times$ *Cdc42*^{2}; *P*=0.007 for $w^{1118} \times w^{1118}$ versus $w^{1118} \times$ *Cdc42*^{2}; *P*=0.001 for $w^{1118} \times w^{1118}$ versus *Cdc42*$^{2} \times w^{1118}$; and *P*=0.179 for *Cdc42*$^{2} \times$ *Cdc42*^{2} versus *Cdc42*$^{2} \times w^{1118}$). 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.

Table 1. Genetic interaction assays between Cdc42 and aPKC and between Cdc42/aPKC/Baz and Arp3

	Genotype							
Orb localization	$Cdc42^{3}$/+	$aPKC^{k06403}$/+	$Cdc42^{3}$/+; $aPKC^{k06403}$/+	$Arp3^{83F}$/+	baz^{xi106}/+	$Cdc42^{3}$/+;; $Arp3^{83F}$/+	$aPKC^{k06403}$/+;; $Arp3^{83F}$/+	baz^{xi106}/+;; $Arp3^{83F}$/+
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.

par-6$^{\Delta 226}$ 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 Tepass, 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 relocalization 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^{xi106} null mutant egg chambers and in hypomorphic $aPKC^{k06403}$ mutant egg chambers. Both the anterolateral enrichment of actin and Cdc42 localization were lost in baz^{xi106} and $aPKC^{k06403}$ 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

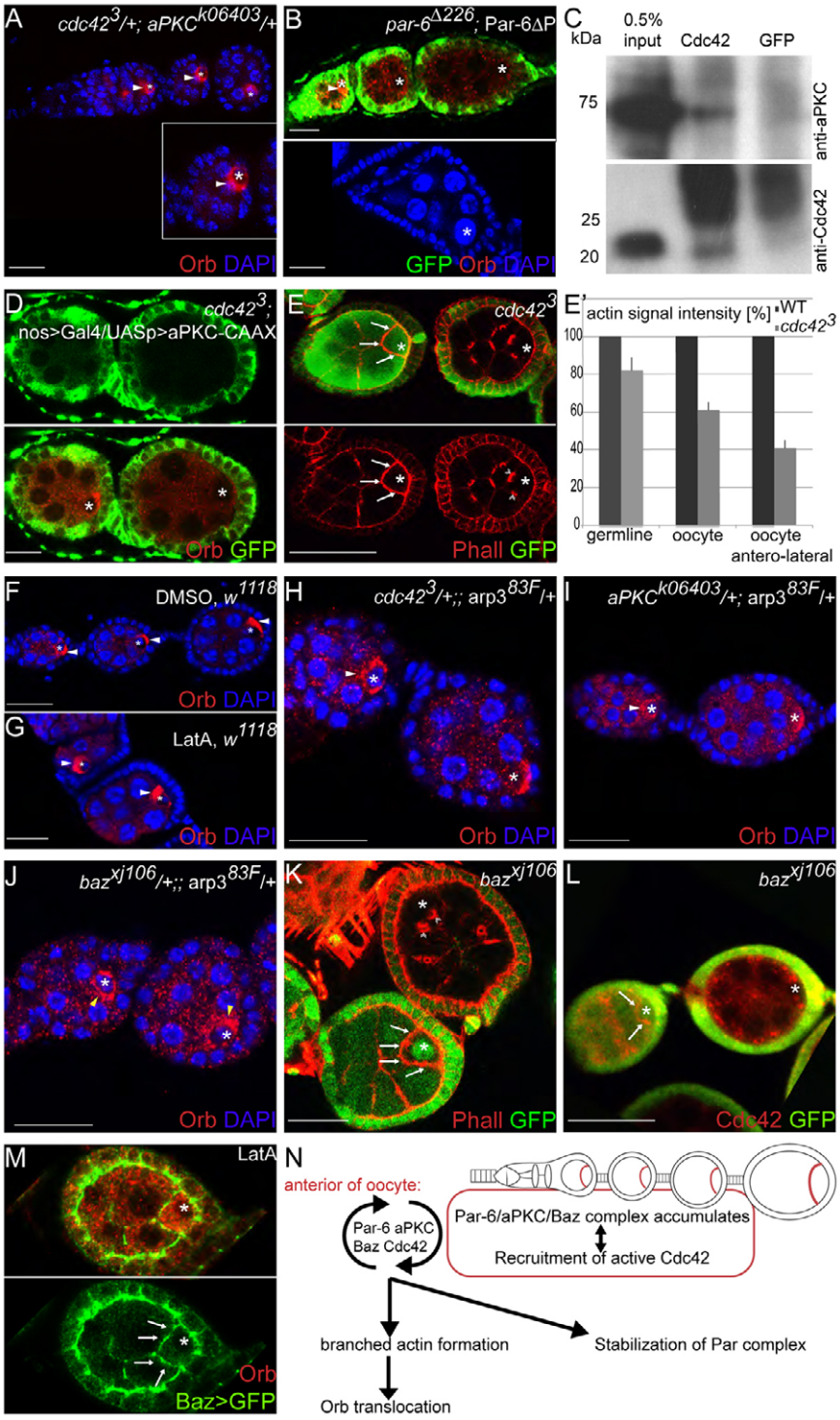

Fig. 4. See next page for legend.

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. Cdc42 and Par proteins cooperate to organize the actin cytoskeleton and establish oocyte polarity. (**A**) *Cdc42*^{3} heterozygous or *aPKC*^{k06403} 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-6ΔP, 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*^{3} 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*^{3} 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. (**E′**) Quantification of Rhodamine-phalloidin staining in wild-type and *Cdc42*^{3} 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. (**F**,**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*^{3}, *aPKC*^{k06403}, *baz*^{xj106} or *Arp3*^{83F} heterozygous oocytes do not lose polarity, whereas transheterozygous oocytes display anteriorly mislocalized Orb protein. (**K**) *baz*^{xj106} 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*^{xj106} 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 polarization of the oocyte, 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, PKCτ/λ 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 Tepass, 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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The authors declare no competing financial interests.

Supplementary material
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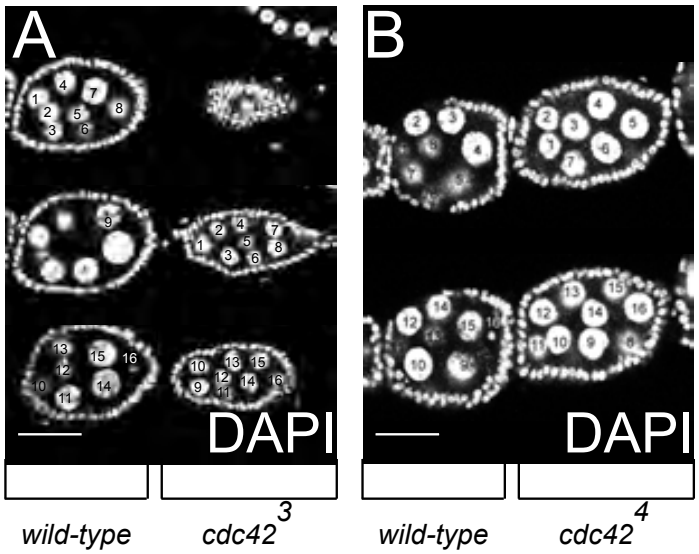

Fig. S1. Nurse cell and oocyte development in *Cdc42*^{3} and *Cdc42*^{4} egg chambers. (**A**) *Cdc42*^{3} mutant egg chambers develop only until stage 5 and display 16 nurse cells. Several stacks are displayed to show all 15 nuclei and oocyte of wild-type egg chamber and the 16 nurse cells of the mutant egg chamber. (**B**) Some *Cdc42*^{4} mutant egg chambers develop until stage 5 and display 16 nurse cells. Two stacks are displayed to show all 15 nuclei and oocyte of the wild-type egg chamber and the 16 nurse cells of the mutant egg chamber. Scale bars: 25 μm.

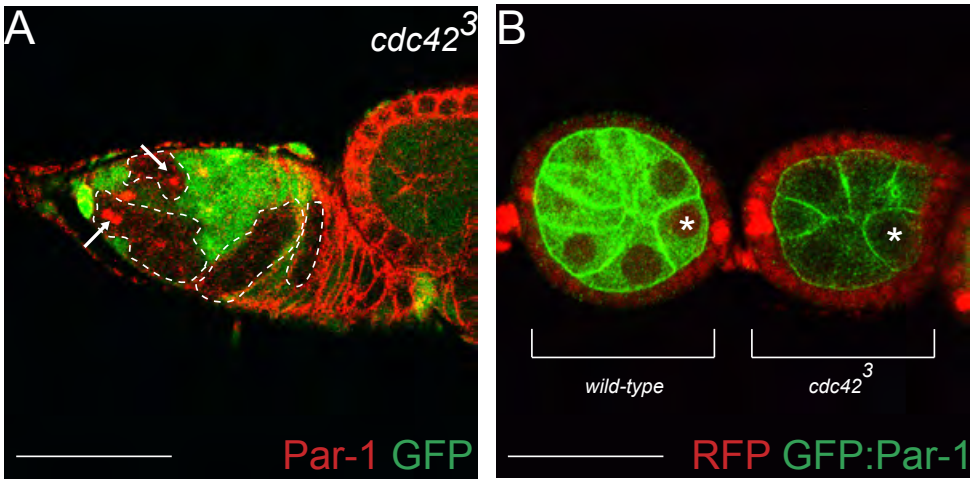

Fig. S2. Par-1 localization is mildly affected by loss of Cdc42. (**A**,**B**) *Cdc42*^{3} mutant clones are marked by loss of nuclear GFP (A) or RFP (B). Par-1 localizes normally to the fusome in mutant clones (A). Mutant clones are encircled with a dashed line. Arrows point to Par-1-positive fusome. GFP-Par-1 localization is weaker in *Cdc42*^{3} germline clones as compared with control egg chambers (B; 20%, *n*=15). Scale bars: 50 μm.

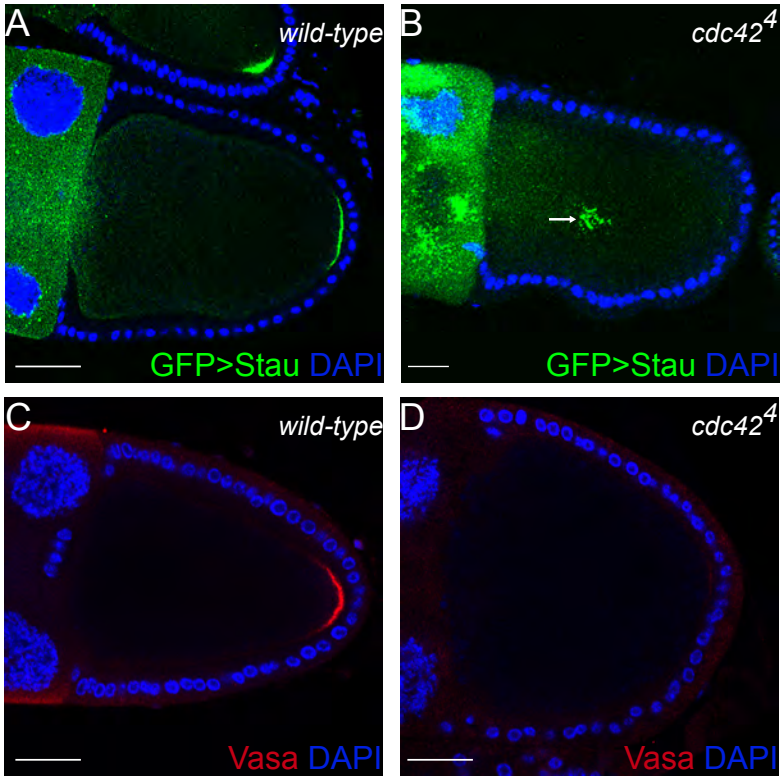

Fig. S3. Staufen and Vasa mislocalize in *Cdc42⁴* germline clones. (**A**,**B**) *Cdc42⁴* mutant clones recovered using the dominant female sterile technique (ovoD) display mislocalized GFP-Staufen protein (green, 25% mislocalization, *n*=118) at stage 9/10 and a mispositioned oocyte nucleus (4% mispositioning, *n*=118). Arrow indicates nucleus and GFP-Staufen localization. (**C**,**D**) *Cdc42⁴* mutant clones recovered using the dominant female sterile technique (ovoD) display mislocalized Vasa (red, 30% mislocalization, *n*=56) at stage 10. Scale bars: 25 μm.

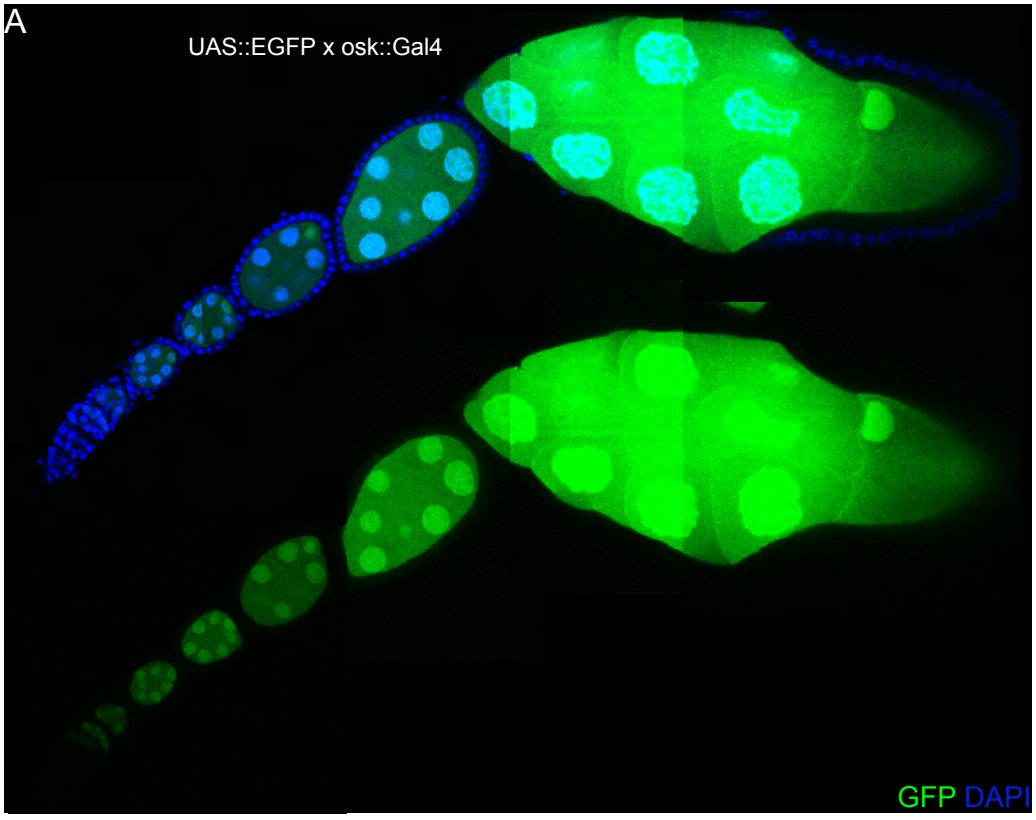

Fig. S4. Activity of the germline driver *oskar>Gal4*. Ovariole from flies expressing UAS>EGFP using the driver *oskar>Gal4*. *oskar>Gal4* drives expression from early to late oogenesis.

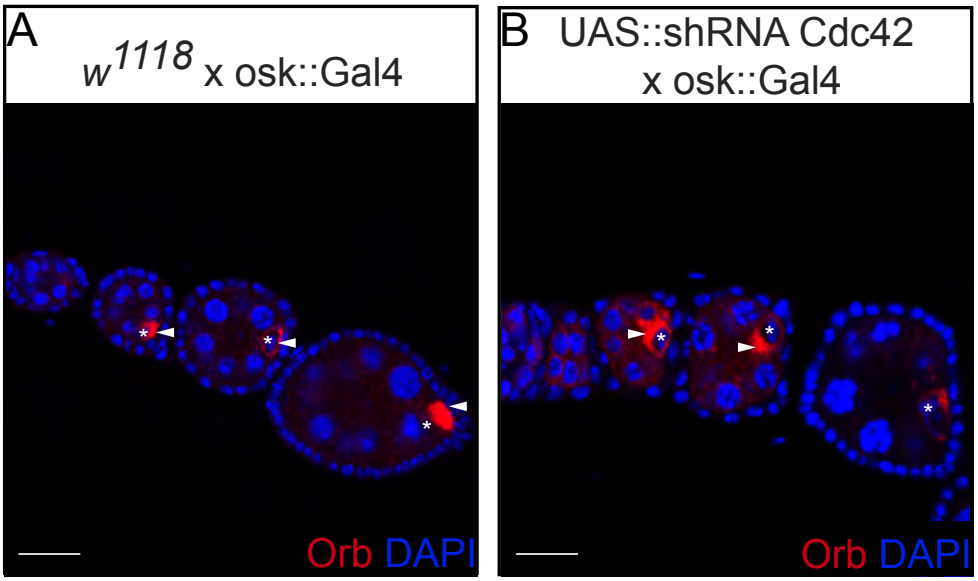

Fig. S5. Knockdown of Cdc42 results in failure of Orb translocation. Orb protein does not translocate from the anterior to the posterior of the oocyte upon knockdown of Cdc42 (26%, *n*=81). (**A**) Control; (**B**) knockdown. Asterisks mark oocytes of interest; arrowheads pointing to the right mark anterior protein localization; arrowheads pointing to the left mark posterior protein localization. Scale bars: 25 μm.

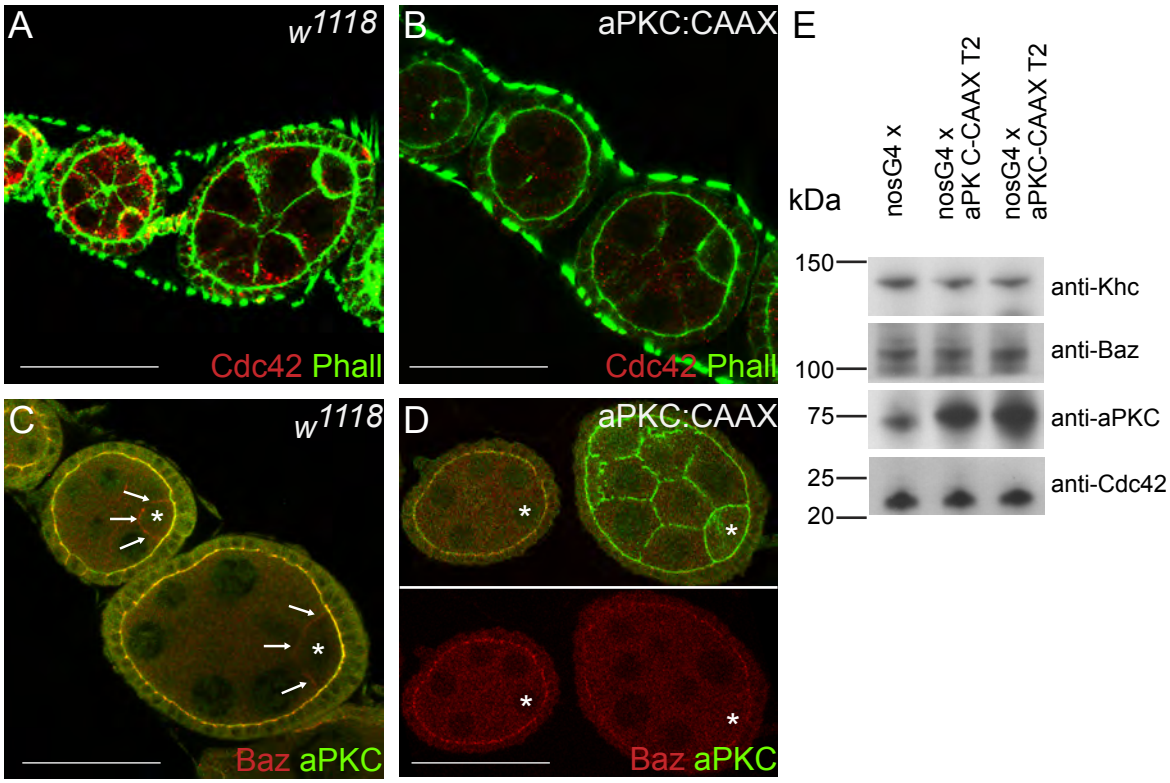

Fig. S6. Overexpression of membrane-tethered aPKC reduces cortical localization of polarity proteins. (**A**,**B**) Cdc42 (red) and actin (green) localization in w^{1118} flies (A) and flies expressing UASp>aPKC-CAAX driven by *nanos>Gal4* (B). (**C**,**D**) Baz (red) and aPKC (green) localization in w^{1118} flies (C) and flies expressing UASp>aPKC-CAAX driven by *nanos>Gal4* (D). Arrows point to specific protein localization. (**E**) Protein levels are not visibly reduced on western blot when membrane-tethered aPKC is expressed in the germline. Scale bars: 25 μm.

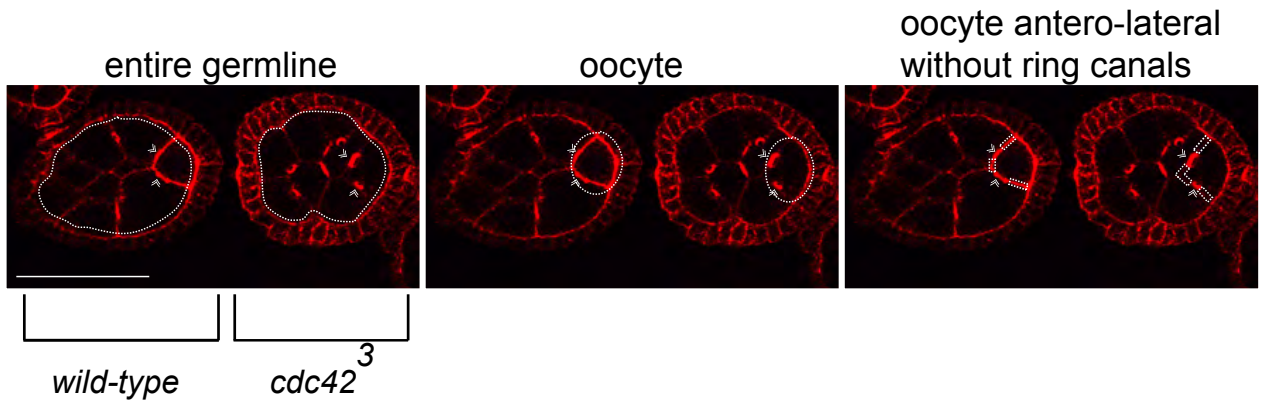

Fig. S7. Analysis of actin levels in mutant and wild-type egg chambers. Actin levels were quantified by measuring the mean intensity of Rhodamine-phalloidin in either the whole germline tissue of the cyst (left image) or the oocyte only (middle image). To consider only regions where Cdc42 localization is enriched, the anterior and lateral regions of oocytes were also analyzed (right image), not taking into account the ring canals (double arrowheads). Note that analysis was always performed on *Cdc42*^{3} mutant egg chambers that could be directly compared with a younger wild-type egg chamber of the same ovariole.

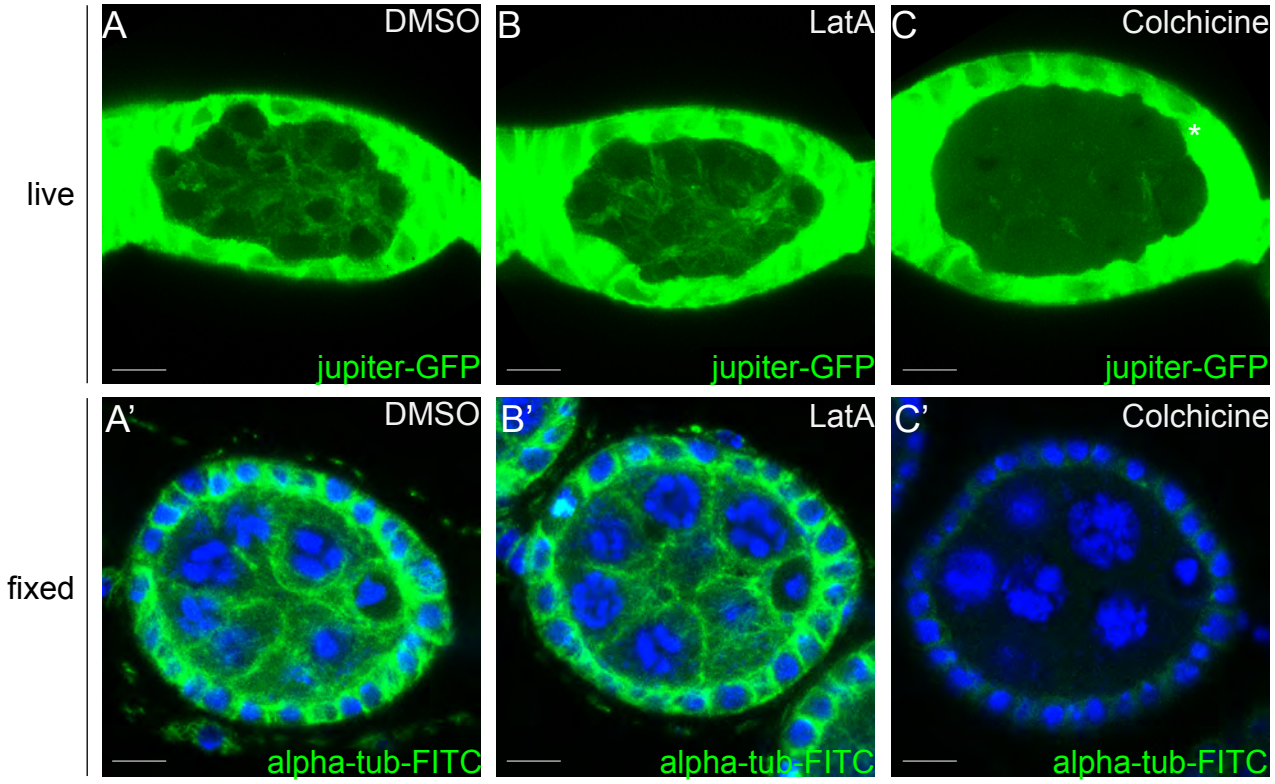

Fig. S8. Latrunculin A treatment does not interfere with microtubules. (**A-C′**) Live imaging (A,B,C; Jupiter-GFP) and fixed imaging (A′,B′,C′; α-tubulin-FITC) of microtubules after drug treatment. Flies were fed with DMSO (1:10; A,A′), 1 mM Latrunculin A (B,B′) or 50 μg/ml Colchicine (C,C′) for 16 hours prior to dissection. Live imaging was performed on Jupiter-GFP flies. Images show maximal projections of 160 images taken with an imaging rate of 1.95 seconds/image. For fixed analysis, w^{1118} flies were used. Scale bars: 25 μm.

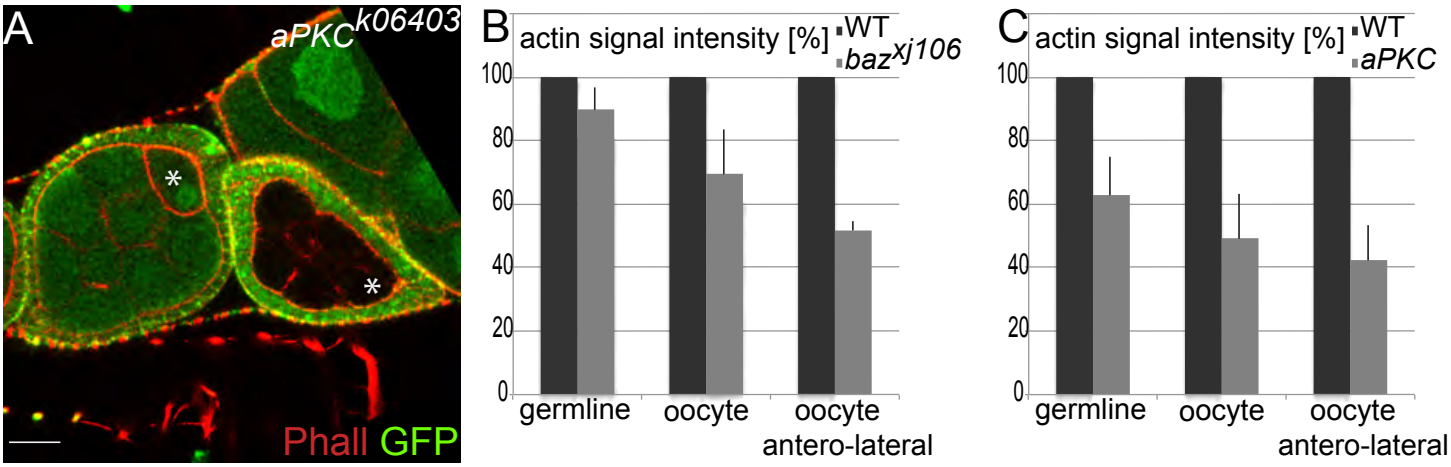

Fig. S9. Cdc42 and Par proteins cooperate to organize the actin cytoskeleton and establish oocyte polarity. (**A**) *aPKC*^{K06403} mutant egg chambers marked by loss of nuclear GFP result in loss of the pronounced actin cytoskeleton at the anterolateral cortex of the oocyte (86%, *n*=7). The actin cytoskeleton is labeled using fluorescently labeled phalloidin. Scale bar: 25 μm. (**B**,**C**) Quantification of phalloidin staining in wild-type, *baz*^{xj106} (B) and *aPKC*^{K06403} (C) mutant egg chambers (left bars), oocytes (middle bars), and the anterior and lateral regions of oocytes (avoiding the posterior, which is in contact with the somatic cells; right bars). Standard error is indicated.

Table S1. Experiments using drugs to specifically inhibit Rho, Rac or Cdc42 were inconclusive

		Drug treatment		
Orb mislocalization	**DMSO**	**Rac1 inhibitor**	**H-1152**	**IPA-3**
Mislocalized at anterior	1	5	6	11
Normal	65	30	30	58
Absent	0	1	0	0
Total	66	36	36	69
Orb mislocalization (%)	**1.5**	**17**	**17**	**16**
Notes				Morphology affected

Inhibition of Rac GTPase with 50 μM Rac inhibitor, inhibition of Rho GTPase with 2 μM H-1152 and inhibition of Cdc42-mediated events with 100 μM IPA-3 treatment for 4 hours did not lead to conclusive results because the morphology of the ovarioles was affected.