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Bazooka is required for polarisation of the *Drosophila* anterior-posterior axis

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SUMMARY

The *Drosophila* anterior-posterior (AP) axis is determined by the polarisation of the stage 9 oocyte and the subsequent localisation of *bicoid* and *oskar* mRNAs to opposite poles of the cell. Oocyte polarity has been proposed to depend on the same PAR proteins that generate AP polarity in *C. elegans*, with a complex of Bazooka (Baz; Par-3), Par-6 and aPKC marking the anterior and lateral cortex, and Par-1 defining the posterior. The function of the Baz complex in oocyte polarity has remained unclear, however, because although *baz*-null mutants block oocyte determination, egg chambers that escape this early arrest usually develop normal polarity at stage 9. Here, we characterise a *baz* allele that produces a penetrant polarity phenotype at stage 9 without affecting oocyte determination, demonstrating that Baz is essential for axis formation. The dynamics of Baz, Par-6 and Par-1 localisation in the oocyte indicate that the axis is not polarised by a cortical contraction as in *C. elegans*, and instead suggest that repolarisation of the oocyte is triggered by posterior inactivation of aPKC or activation of Par-1. This initial asymmetry is then reinforced by mutual inhibition between the anterior Baz complex and posterior Par-1 and Lgl. Finally, we show that mutation of the aPKC phosphorylation site in Par-1 results in the uniform cortical localisation of Par-1 and the loss of cortical microtubules. Since non-phosphorylatable Par-1 is epistatic to uninhibitable Baz, Par-1 seems to function downstream of the other PAR proteins to polarise the oocyte microtubule cytoskeleton.

KEY WORDS: Axis formation, Par-1, Par-6, Lgl, Polarity, Oogenesis, *Drosophila*

INTRODUCTION

The primary body axis of many organisms is already specified at the one-cell stage through the localisation of cytoplasmic determinants to opposite sides of the cell. The formation of the axis therefore depends on upstream cues that lead to the polarisation of the egg and the subsequent targeting of the determinants to the appropriate positions.

This process has been studied in detail in *C. elegans* and *Drosophila*, which set up their anterior-posterior (AP) axes at different stages of development in response to different cues. Nevertheless, the same conserved PAR proteins appear to play an essential role in the establishment of polarity in each system (Goldstein and Macara, 2007).

The *C. elegans* AP axis becomes polarised at fertilisation in response to the entry of the sperm, which triggers a loss of cortical actin and myosin II at the posterior, leading to a contraction of the remaining actomyosin cortex towards the anterior (Cheeks et al., 2004; Goldstein and Hird, 1996; Munro et al., 2004). This leads to an anterior restriction of the PAR-3 complex [PAR-3, PAR-6 and aPKC (PKC-3 – WormBase)], allowing the posterior recruitment of PAR-1 and PAR-2 (Cheeks et al., 2004; Cuenca et al., 2003; Munro et al., 2004). As a result, the PAR proteins form complementary cortical domains that are maintained by mutual antagonism. PAR-2 excludes the PAR-3 complex from the posterior, while aPKC phosphorylates PAR-1 and PAR-2 to prevent their association with the anterior cortex (Gonczy and Rose, 2005; Hao et al., 2006). The polarised arrangement of PAR proteins controls all subsequent asymmetries in the one-cell zygote (Cowan and Hyman, 2007; Gonczy and Rose, 2005).

Unlike *C. elegans*, both axes in *Drosophila* are defined during the development of the oocyte. Oogenesis begins when a germline stem cell divides asymmetrically to produce a daughter stem cell and a cystoblast, which then undergoes four mitoses to produce a cyst of 16 germ cells that are connected by cytoplasmic bridges called ring canals (Spradling, 1993). The germline cyst is then enveloped by a monolayer of somatic follicle cells to form an egg chamber. At this stage, the first polarity in the cyst becomes apparent with the formation of a polarised microtubule cytoskeleton that extends throughout all 16 cells of the cyst (Theurkauf et al., 1993). The microtubule network focuses minus-end-directed transport of oocyte-specific factors towards one cell, which will become the oocyte; the remaining 15 cells become nurse cells (Bastock and St Johnston, 2008).

Oocyte specification depends on the *Drosophila* orthologues of the *C. elegans par* genes, with the exception of *par-2*, which is not conserved in *Drosophila*. In wild-type egg chambers, oocyte-specific factors and microtubule minus ends first accumulate at the anterior of the prospective oocyte, and then translocate to the posterior of the cell, where a stable microtubule-organising centre (MTOC) forms (Huynh and St Johnston, 2000). This anterior-to-posterior translocation does not occur in *par-1*, *bazooka* (*baz*; the *par-3* orthologue), *par-6* and *aPKC* mutants, however, and the oocyte reverts to the nurse cell fate (Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b).

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The AP axis of the fly is specified in mid-oogenesis, when the posterior follicle cells signal to induce a new polarity in the oocyte (Ruohola et al., 1991). The original posterior MTOC is disassembled and new microtubules are nucleated or anchored at the anterior and lateral cortex, resulting in the formation of an AP gradient, in which most microtubule minus ends lie at the anterior with their plus ends extending towards the posterior pole (Cha et al., 2002; Clark et al., 1994; Clark et al., 1997; Theurkauf et al., 1992). This polarised microtubule cytoskeleton then defines the AP axis by directing the localisation of *bicoid* (*bcd*) and *oskar* (*osk*) mRNAs to opposite poles of the cell (St Johnston, 2005).

As in *C. elegans*, PAR proteins seem to play a central role in the repolarisation of the *Drosophila* oocyte that defines the AP axis of the embryo. The earliest known marker for this polarity is the recruitment of a GFP-Par-1 fusion protein to the posterior cortex of the oocyte, while a Baz-GFP fusion defines a complementary anterior and lateral cortical domain (Benton and St Johnston, 2003b; Doerflinger et al., 2006). Furthermore, strong *par-1* hypomorphs lead to a fully penetrant polarity defect, in which *osk* mRNA localises to the centre of the oocyte, whereas *bcd* mRNA extends around the cortex (Shulman et al., 2000; Tomancak et al., 2000). The opposing anterior/lateral and posterior domains also appear to be maintained by mutual antagonism between Par-1 and the tripartite Baz–Par-6–aPKC complex. Par-1 phosphorylates two conserved serines in Baz to disrupt its ability to oligomerise and interact with aPKC, and a non-phosphorylatable form of Baz localises to the posterior cortex and disrupts oocyte polarity (Benton and St Johnston, 2003b). Conversely, mammalian aPKC (PKC zeta) has been shown to phosphorylate a conserved site in PAR-1, and mutation of five amino acids in this domain of *Drosophila* Par-1 leads to its mislocalisation around the anterior and lateral cortex, suggesting that aPKC phosphorylation of this site might exclude Par-1 from this region (Doerflinger et al., 2006; Hurov et al., 2004; Suzuki et al., 2004).

Although the available evidence suggests that the PAR proteins play a similar role in the polarisation of the AP axis of *Drosophila* as they do in *C. elegans*, a number of important unanswered questions remain. First, there is no direct evidence that Baz, Par-6 or aPKC are required for oocyte polarity in mid-oogenesis. Germline clones with null mutations in these genes are blocked at the initial polarisation of the oocyte that is required for the maintenance of oocyte fate, and it is therefore difficult to examine their function at later stages of oogenesis (Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b). Nevertheless, some mutant egg chambers escape this early arrest and most of these go on to develop normally (Benton et al., 2002; Doerflinger et al., 2006; Kuchinke et al., 1998; Tian and Deng, 2008). Furthermore, germline clones with hypomorphic mutations in aPKC that reduce or abolish its kinase activity show no effect on AP axis formation (Kim et al., 2009).

Second, it is unclear how this cortical PAR polarity is established. It has recently been proposed that Lethal (2) giant larvae [Lgl] functions as an upstream factor that allows Par-1 to localise to the oocyte posterior cortex (Tian and Deng, 2008). This key role of Lgl is hard to reconcile, however, with the observation that *lgl*-null germline clones have no phenotype and with the suggestion that the polarity defects only occur when posterior follicle cells are also mutant (Li et al., 2008).

Finally, almost nothing is known about how the polarised arrangement of cortical PAR proteins regulates the organisation of the microtubule cytoskeleton to determine the destinations of *bcd* and *osk* mRNAs and hence the AP axis.

Here, we report the identification of a novel *baz* allele that specifically disrupts AP axis formation without affecting oocyte determination, indicating that Baz is required for this process. We also investigate the relationships between the PAR proteins and Lgl in the polarisation of the oocyte and analyse how these cortical polarity factors control microtubule organisation.

MATERIALS AND METHODS

Fly stocks

Wild-type stocks were Oregon R* or w^{1118}. Transgenes, aberrations or mutant alleles were baz^{4} (Muller and Wieschaus, 1996), baz^{EH171} (Eberl and Hilliker, 1988), baz^{815} (Djiane et al., 2005), $baz^{358\text{-}12}$ (this study), $par\text{-}1^{W3}$ and $par\text{-}1^{6323}$ (Shulman et al., 2000), mat-tub-GFP-Par-1 and UASp-GFP:Par-1(N1S)-GFP (Huynh et al., 2001b), UASp-GFP-Par-1^{T786A} (this study), Par-1 protein trap (Lighthouse et al., 2008), UASp-Baz-GFP and UASp-$Baz^{S151AS1085A}$-GFP (Benton and St Johnston, 2003b), UASp-Par-6-Cherry (this study), UASp-Lgl-GFP (Tian and Deng, 2008), the Kin:βgal transgenes, KZ32 and KZ503 (Clark et al., 1994), $Dp(1;4)r^{+}l$ (Bloomington Stock Center), 998/12 (González-Reyes and St Johnston, 1998) and 5A7 (Roth et al., 1995). Gal4 drivers were mat-α4tub:Gal4, mat-α4tub:Gal4-VP16 and nanos:Gal4. Germline clones were generated with FRT 9-2 ovoD and FRT 9-2 GFP (Bloomington Stock Center) using the Flp/ovoD system (Chou and Perrimon, 1992).

Screening procedure

The F_1 screen was designed to identify new mutants that disrupt *bcd* and/or *osk* RNA localisation during oogenesis as described (Luschnig et al., 2004; Schnorrer et al., 2002), with the modification that we established lines from females giving rise to eggs with early-arrest phenotypes, but also with other strong developmental defects. Mutations were induced on a *w f hs-Flp122 FRT9-2* chromosome.

Molecular biology

The *baz* coding region was sequenced by amplifying suitable fragments from genomic DNA of ovaries from germline clones induced with the dominant female sterile (DFS) method. The Par-1^{T786A} mutation was generated by oligonucleotide-directed mutagenesis using the pUASp-GFP-Par-1(N1S) construct (Doerflinger et al., 2006). The Par-6-Cherry fusion protein was produced by cloning mCherry in frame at the C-terminus of the Par-6 coding sequence in the pUASP vector (Rorth, 1998).

Protein biochemistry

Ovarian extracts were prepared as described (Schnorrer et al., 2002). Western blots were probed with rabbit anti-Baz N-terminus at 1:2000 (Wodarz et al., 1999), followed by HRP-linked goat anti-rabbit at 1:5000 (Dianova).

Cytology

In situ hybridisations were performed as described (Doerflinger et al., 2006; Schnorrer et al., 2002). Oocyte microtubules were stained as described (Theurkauf et al., 1992) using an FITC-coupled anti-α-Tubulin antibody at 1:200 (Sigma).

Wheat germ agglutinin (WGA) stainings were performed by incubating fixed ovaries in 1:200 WGA conjugated with Texas Red (Invitrogen) for 20 minutes and washing three times for 10 minutes each.

Antibodies used were rabbit anti-Baz at 1:200 (Wodarz et al., 1999), rabbit anti-Staufen at 1:200 (St Johnston et al., 1991), mouse anti-Orb (sera 4H8 and 6H4 mixed together) at 1:200 (DSHB), rabbit anti-Par-6 at 1:500 (Pinheiro and Montell, 2004) and mouse anti-Gurken (1D12) at 1:200 (DSHB). FITC- and Texas Red-conjugated secondary antibodies (Molecular Probes) were used at 1:100.

RESULTS

Identification of a new *bazooka* allele

We performed a large-scale F_1 germline clone screen on the X chromosome for mutations that disrupt the patterning of the *Drosophila* embryo following the procedure of Luschnig et al. (Luschnig et al., 2004). One of the mutations from this screen,

X-358-12, produced a high frequency of eggs with fused and/or misplaced dorsal appendages, most of which did not develop (data not shown). In the rare cases in which these germline clone eggs developed further, the resulting embryos showed large holes in their cuticles.

We mapped the *X-358-12* mutation to the cytological region 15B1-16A1 using duplications to complement the lethality associated with the phenotype. In complementation tests with alleles of candidate genes in this interval, *X-358-12* was lethal over a null mutation in *bazooka*, baz^{EH171}, and a strong loss-of-function allele, baz^{815}, indicating that it is a novel allele of the locus. Consistent with this, the *baz* coding region of *X-358-12* contains a nonsense mutation not present on the starting chromosome that changes Gln849 into a stop codon (Fig. 1A). We therefore renamed *X-358-12* as baz^{358-12}.

The baz^{358-12} mutation is predicted to give rise to a truncated protein of 90 kDa. Western blots of ovarian extracts prepared from mutant germline clones showed a band of this size, confirming that the mutation gives rise to a stable truncated protein (Fig. 1B). Baz is a large scaffolding protein that contains three conserved regions

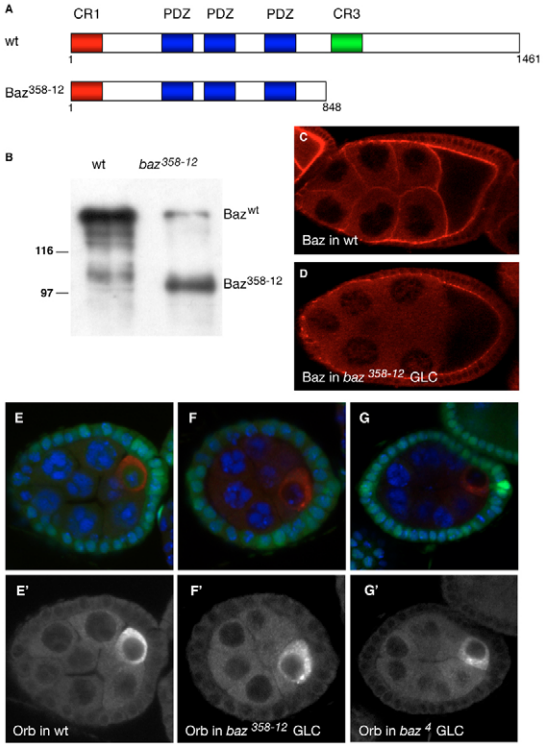

Fig. 1. Identification of a new *bazooka* allele. (**A**) The wild-type (wt) *Drosophila* Baz protein contains three conserved regions (CRs). CR1 (red) mediates the oligomerisation of Baz. CR2 (not labelled) contains three PDZ domains (blue) that interact with Par-6, Pten and adherens junction components. CR3 (green) is a binding site for aPKC. Baz^{358-12} is truncated at amino acid 849, removing the CR3 domain and the rest of the C-terminus. (**B**) Western blot of ovarian extracts probed with anti-Baz antibody. Wild-type Baz is represented by a band of 190 kDa, whereas mutant germline clones show a novel band of the expected size (90 kDa) of the truncated protein. (**C**,**D**) Immunolocalisation of Baz in stage 9 wild-type (C) and baz^{358-12} (D) germline clones. (**E-G'**) Localisation of Orb (red; E′-G′) in a stage 3 wild-type egg chamber (E,E′), in a baz^{358-12} germline clone (F,F′) marked by the loss of nuclear GFP (green) and in a baz^{4} germline clone (G,G′). DNA is stained with DAPI (blue).

called CR1-3 (Fig. 1A). CR1 mediates the oligomerisation of Baz with itself (Benton and St Johnston, 2003a), CR2 contains three PDZ domains that interact with Par-6, Pten and the adherens junction components Echinoid and Armadillo (Lin et al., 2000; von Stein et al., 2005; Wei et al., 2005; Wodarz, 2002), whereas CR3 is a binding site for aPKC (Benton and St Johnston, 2003b; Nagai-Tamai et al., 2002). Since the nonsense mutation in baz^{358-12} occurs N-terminal of CR3, the truncated protein lacks this domain together with the rest of the C-terminus.

Germline clones of all *baz* alleles that have been analysed so far give rise to egg chambers that arrest development at stages 4-5 and contain 16 nurse cells and no oocyte, although a few egg chambers reach late stages when the clones are generated using the FLP/ovoD system. By contrast, baz^{358-12} FLP/ovoD clones of baz^{358-12} produced a normal frequency of late-stage egg chambers, suggesting that this mutation does not affect Baz function in early oogenesis. To test this directly, we generated baz^{358-12} germline clones that were marked by the loss of nuclear GFP expression. These clones showed a wild-type localisation of Orb to the posterior of the oocyte at stage 3, and always contained a differentiated oocyte at later stages. By contrast, in clones of the null allele baz^{4}, Orb localised only transiently to the anterior of the oocyte before this cell reverted to the nurse cell fate (Fig. 1E-G). Thus, the C-terminal region of Baz that includes CR3 is not required for the initial polarisation of the oocyte or for the maintenance of oocyte fate.

The survival of baz^{358-12} germline clones to later stages allowed us to examine the localisation of the truncated Baz protein when the oocyte is repolarised during stages 7-9. In wild-type egg chambers, Baz is localised at the anterior and lateral cortex of the oocyte, as well as at the cortex of the nurse cells and the apical sides of the follicle cells (Fig. 1C,D) (Benton and St Johnston, 2003a). baz^{358-12} germline clones, by contrast, showed no Baz localisation at the anterior cortex of the oocyte or at the cortex of the nurse cells (we could not determine whether there was any staining at the lateral cortex of mutant oocytes because this signal cannot be distinguished from the apical staining in the adjacent wild-type follicle cells) (Fig. 1D). The cortical localisation of Baz in mid-oogenesis therefore depends on its C-terminal domain.

Baz is required for nuclear positioning and AP polarity

Since baz^{358-12} mutant eggs have fused and misplaced dorsal appendages, we examined the localisation of Gurken protein in stage 9 mutant germline clones. In wild-type oocytes, the nucleus migrates to the anterior/dorsal corner at stage 7, and *gurken* mRNA and protein localise above the nucleus where Gurken signals to induce dorsal fates in the overlying follicle cells (Neuman-Silberberg and Schüpbach, 1993). The oocyte nucleus was mislocalised in 75% of stage 9/10 baz^{358-12} germline clones (n=126), and Gurken mislocalised with the nucleus (Fig. 2B,I,J). This defect therefore probably accounts for the misplaced and fused dorsal appendages of the mutant eggs.

The migration of the nucleus depends on the AP polarisation of the oocyte, and we therefore examined whether the localisation of *bcd* and *osk* mRNAs was altered in baz^{358-12} germline clones. *bcd* mRNA is initially localised in a ring at the anterior cortex of wild-type oocytes and relocalises into a disc during stage 10b (Fig. 2A). *bcd* mRNA was found at random positions at the cortex of baz^{358-12} mutant oocytes but usually adjacent to the mislocalised oocyte nucleus (Fig. 2B and data not shown). In wild-type oocytes, *osk* mRNA is localised at the posterior pole from stage 9 onwards (Fig. 2C) (Ephrussi et al., 1991; Kim-Ha et al., 1991). In baz^{358-12} mutant

oocytes, *osk* mRNA was either diffuse, detached from the posterior or localised to a dot in the centre of the oocyte at stages 9-10A (Fig. 2D and data not shown). We also observed a similar phenotype for Staufen, an RNA-binding protein that colocalises with *osk* mRNA (98% at stage 10, n=140) (Fig. 2K,L) (St Johnston et al., 1991).

To prove that the $baz^{358\text{-}12}$ phenotype is caused by a loss of Baz function and is not due to some dominant-negative effect of truncated Baz, we also generated germline clones of a strong *baz* allele, baz^{4}, and examined the phenotypes of the rare escapers that developed to stage 10. Of the escapers, 35% (n=52) showed polarity defects, which ranged from weak phenotypes, in which some Staufen was mislocalised, to stronger defects that were similar to those produced by $baz^{358\text{-}12}$ (data not shown).

Since the repolarisation of the oocyte depends on signalling from the posterior follicle cells, we also examined whether these cells are correctly specified in $baz^{358\text{-}12}$ germline clones. These cells differentiated normally and expressed the 998/12 enhancer-trap, which is a specific marker for posterior follicle cells (González-Reyes and St Johnston, 1998) (Fig. 2G,H). In addition, these cells did not express the 5A7 enhancer-trap, which is a border cell marker, indicating that they have responded normally to Gurken signalling and have adopted a posterior, rather than an anterior, fate (Gonzalez-Reyes et al., 1995; Roth et al., 1995) (Fig. 2E,F). Thus, Baz appears to be specifically required in the oocyte for the establishment of AP polarity at mid-oogenesis.

Since *bcd* and *osk* mRNA localisation and the positioning of the nucleus are microtubule dependent, we examined whether $baz^{358\text{-}12}$ mutants disrupt the oocyte microtubule cytoskeleton. The posterior bias in the distribution of microtubule plus ends can be examined using a fusion between the motor domain of the plus-end-directed motor protein Kinesin and β-galactosidase (Kin:βgal), which accumulates at the posterior of wild-type oocytes at stage 9 (Fig. 3A) (Clark et al., 1994). Kin:βgal was not properly localised in $baz^{358\text{-}12}$ mutant oocytes, suggesting that microtubule plus ends are not enriched at the oocyte posterior (Fig. 3B). Endogenous Kinesin heavy chain also failed to localise normally to the posterior of $baz^{358\text{-}12}$ mutant oocytes (data not shown) (Palacios and St Johnston, 2002).

Microtubules are distributed in a weak anterior-to-posterior gradient in wild-type stage 7-10A oocytes (Fig. 3C). By contrast, the microtubules showed a fairly uniform distribution in $baz^{358\text{-}12}$ mutant oocytes, with a higher density near the mislocalised oocyte nucleus and around the cortex (Fig. 3D).

These results indicate that, like Par-1, Baz functions upstream of the microtubule cytoskeleton in the polarisation of the oocyte. We therefore investigated whether *baz* is also required for the posterior recruitment of Par-1, using a GFP-Par-1 transgenic line (Shulman et al., 2000). In wild-type oocytes, GFP-Par-1 formed a crescent at the posterior cortex at stage 9 (Fig. 3E). In $baz^{358\text{-}12}$ mutant oocytes, however, GFP-Par-1 showed a fairly uniform distribution around the oocyte cortex (Fig. 3F). Thus, $baz^{358\text{-}12}$ disrupts all aspects of oocyte polarity, suggesting that it plays an early and essential role in this process.

Dynamic localisation of Baz and Par-6

In most polarised cell types, Baz functions in a complex with Par-6 and aPKC and shows a complementary localisation to Par-1. We therefore investigated the relationship between the PAR proteins in the oocyte and how they become asymmetrically localised by analysing the distributions of GFP-tagged versions of Baz and Par-1 and Par-6-Cherry from stage 7 of oogenesis until stage 9.

The first sign of oocyte repolarisation is the recruitment of GFP-Par-1 to the posterior cortex, which occurs shortly before the nucleus migrates to the anterior during stage 7 (Fig. 4A, arrows) (Doerflinger et al., 2006). At this stage, Baz-GFP also localises to the posterior, and overlaps with the Par-1 domain (Fig. 4C, arrows). Par-6-Cherry,

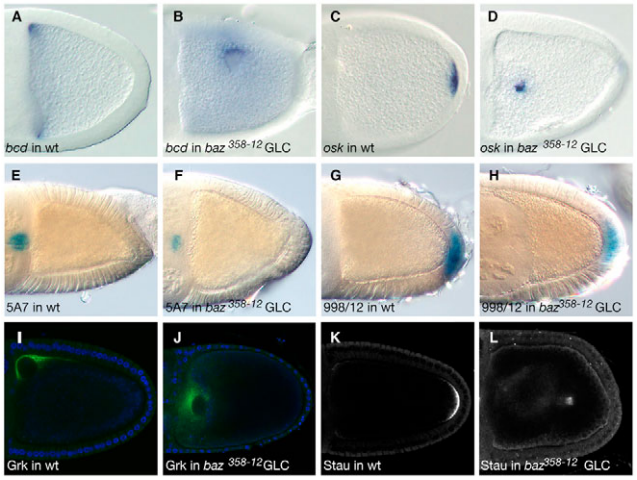

Fig. 2. $baz^{358\text{-}12}$ disrupts polarisation of the AP axis in the stage 9 oocyte. (**A**,**B**) *bcd* mRNA localisation in stage 9 wild-type (A) and $baz^{358\text{-}12}$ (B) oocytes. (**C**,**D**) *osk* mRNA localisation in stage 9 wild-type (C) and $baz^{358\text{-}12}$ (D) oocytes. (**E**,**F**) Expression of the 5A7 enhancer-trap, which is a border cell marker, in stage 10 wild-type (E) and $baz^{358\text{-}12}$ (F) oocytes. (**G**,**H**) Expression of the posterior follicle cell-specific 998/12 enhancer-trap in stage 9 wild-type (G) and $baz^{358\text{-}12}$ (H) oocytes. (**I**,**J**) Gurken protein (green) localisation in stage 10 wild-type (I) and $baz^{358\text{-}12}$ mutant (J) oocytes. (**K**,**L**) Staufen localisation in stage 10 wild-type (K) and a $baz^{358\text{-}12}$ (L) oocytes.

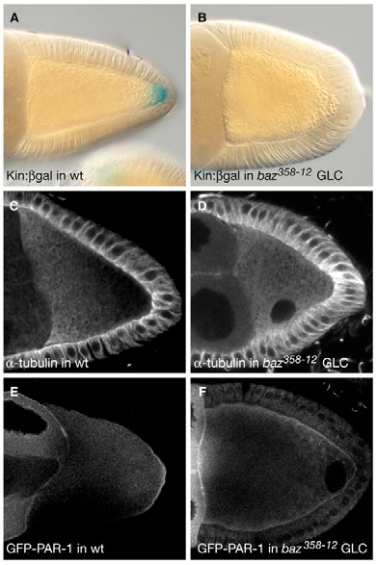

Fig. 3. Par-1 is mislocalised in $baz^{358\text{-}12}$ oocytes. (**A**,**B**) Kinesin:βgal in stage 10 wild-type (A) and $baz^{358\text{-}12}$ (B) oocytes. (**C**,**D**) Microtubules stained with α-Tubulin-FITC in stage 9 wild-type (C) and $baz^{358\text{-}12}$ (D) oocytes. (**E**,**F**) GFP-Par-1 in stage 10 wild-type (E) and $baz^{358\text{-}12}$ (F) oocytes.

by contrast, localised around the entire cortex with slightly lower levels at the posterior than elsewhere (Fig. 4B, arrows). The posterior crescent of Par-1 strengthened and expanded during stages 8 and 9 (Fig. 4D,G). Baz still overlapped Par-1 during stage 8 and extended more anteriorly, but was excluded from the most anterior region of the lateral cortex (Fig. 4F). At this stage, Par-6 had disappeared from the posterior cortex and formed a complementary anterior/lateral domain to the posterior Par-1 domain (Fig. 4E, arrows). Baz finally disappeared from the posterior at stage 9, as previously reported (Benton and St Johnston, 2003b), and both Par-6 and Baz showed complementary localisations to Par-1 (Fig. 4G-I).

Since Par-6 and Baz disappeared from the posterior at different stages, we also asked whether the posterior exclusion of both proteins is Par-1-dependent. In the strongest viable *par-1* hypomorphic mutant combination, *par-1*^{6323}/*par-1*^{W3}, both Baz and Par-6 showed a uniform cortical localisation at stage 9 (Fig. 4K,L). We also observed a similar uniform distribution of Par-6-Cherry in oocytes expressing Baz$^{S151A\ S1085A}$-GFP, in which Baz cannot be phosphorylated by Par-1 and therefore covers the entire cortex (Fig. 4M and data not shown). The posterior exclusion of Par-6 therefore depends on Baz phosphorylation, indicating that Par-6 is recruited to the cortex by binding to Baz. Consistent with this, endogenous Par-6 can be detected along the anterior cortex of wild-type oocytes, and this localisation was lost in *baz*$^{358\text{-}12}$ mutant clones (Fig. 4N, arrow; Fig. 4O). These results support a model in which Par-1 and the Baz–Par-6–aPKC complex antagonise each other to establish complementary posterior and anterior/lateral domains. However, this PAR protein asymmetry is dynamic during stages 7-8, and only reaches its final form at stage 9, coincident with the onset of *osk* mRNA localisation to the posterior pole.

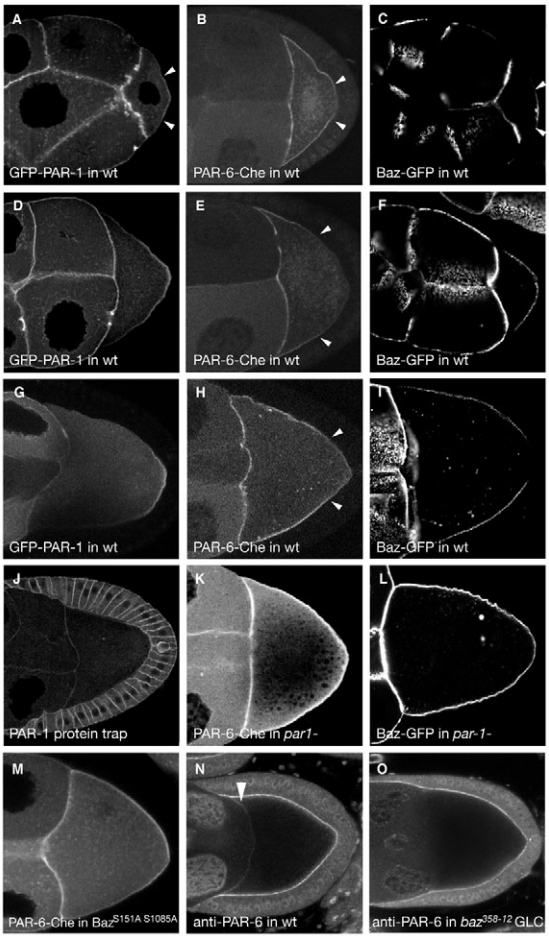

Fig. 4. Dynamic localisation patterns of fluorescently tagged Par-1, Par-6 and Baz during stages 7-9. (**A**,**D**,**G**) GFP-Par-1(N1S) in stage 7 (A), 8 (D) and 9 (G) wild-type oocytes. (**B**,**E**,**H**) Par-6-Cherry in stage 7 (B), 8 (E) and 9 (H) wild-type oocytes. (**C**,**F**,**I**) Baz-GFP in stage 7 (C), 8 (F) and 9 (I) wild-type oocytes. (**J**) Expression of the Par-1-GFP protein-trap line in a wild-type oocyte at stage 9. (**K**) Par-6-Cherry in a *par-1*^{6323}/*par-1*^{W3} stage 9 oocyte. (**L**) Baz-GFP in a *par-1*^{6323}/*par-1*^{W3} stage 9 oocyte. (**M**) Par-6-Cherry in a stage 9 oocyte expressing Baz$^{S151A\ S1085A}$-GFP. (**N**,**O**) Immunostaining of endogenous Par-6 in stage 9 wild-type (N) and *baz*$^{358\text{-}12}$ (O) oocytes.

The observations above are mainly based on overexpressed fluorescently tagged fusion proteins as it is not possible to visualise the endogenous proteins in the oocyte by antibody staining, either because of the strong apical signal in the follicle cells (Baz and Par-6) or because the available antibodies do not label the cortical population of Par-1 (Doerflinger et al., 2006). Thus, it is possible that some of these protein distributions might be affected by overexpression. To address this issue, we examined the localisation of a Par-1 protein-trap line that produces functional Par-1 protein with internal GFP under the control of its endogenous regulatory elements and is therefore expressed at wild-type levels. The 'trapped' Par-1 protein also localised to the posterior cortex of the oocyte at stage 9, indicating that this enrichment is not an artefact of the overexpression of the UAS transgenes (Fig. 4J).

The posterior localisation of Lgl is reduced in *par-1* hypomorphic mutant oocytes

Lgl has been reported to localise to the posterior at stage 6 of oogenesis and to act upstream of Par-1 in the establishment of the AP axis in the oocyte, although this result is controversial (Li et al., 2008; Tian and Deng, 2008). We therefore compared the localisation of Lgl-GFP with that of GFP-Par-1 during stages 6-9 of oogenesis. Unlike GFP-Par-1, we could never detect Lgl-GFP at the posterior cortex of the oocyte when the oocyte nucleus was still at the posterior (Fig. 5A,B, arrows). Lgl-GFP began to appear at the posterior during late stage 8 and early stage 9 and formed a broad posterior crescent at stage 9 that extended more anteriorly than the GFP-Par-1 crescent (Fig. 5C). Thus, Lgl would seem to localise too late to play a role in the initial recruitment of Par-1 to the posterior, although it might help to stabilise this localisation at later stages. In support of this view, we observed that GFP-Par-1 is still enriched at the posterior of the oocyte in germline clones of an *lgl*-null allele, *lgl*^{4}, although the levels were much lower than in wild type (Fig. 5D) (Tian and Deng, 2008).

To test whether Par-1 plays a reciprocal role in Lgl localisation, we expressed the Lgl-GFP transgene in a strong *par-1* hypomorphic mutant combination. Lgl-GFP was still localised at the posterior of *par-1*^{6323}/*par-1*^{W3} mutant oocytes, but at much lower levels than in the wild type (Fig. 5E,E′). Thus, Lgl and Par-1 seem to mutually reinforce each other's posterior localisation, perhaps by inhibiting the Baz–Par-6–aPKC complex by parallel mechanisms.

Mutation of a conserved aPKC phosphorylation site in Par-1 disrupts its localisation and oocyte polarity

Exclusion of Par-1 from the anterior and lateral cortex depends on a conserved 16 amino acid motif in the linker domain, which contains a threonine that has been shown to be phosphorylated by aPKC in mammalian cells (Doerflinger et al., 2006; Hurov et al., 2004;

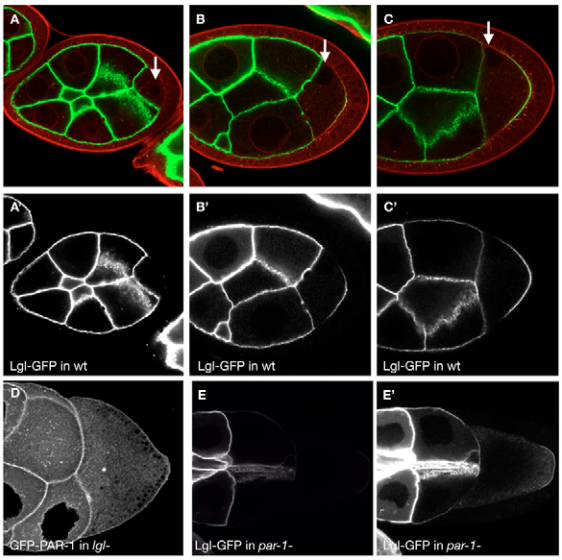

Fig. 5. The posterior recruitment of Lgl depends on Par-1. (**A-C′**) Lgl-GFP at stages 7 (A,A′), 8 (B,B′) and 9 (C,C′) of oogenesis. Wheat germ agglutinin (WGA) labels the membranes (red). The arrows in A-C indicate the position of the oocyte nucleus. (**D**) GFP-Par-1 in a stage 9 *lgl*^{4} oocyte. (**E**,**E′**) Lgl-GFP in stage 9 *par-1*^{6323}/*par-1*^{W3} oocyte. E, normal laser power; E′, high laser power.

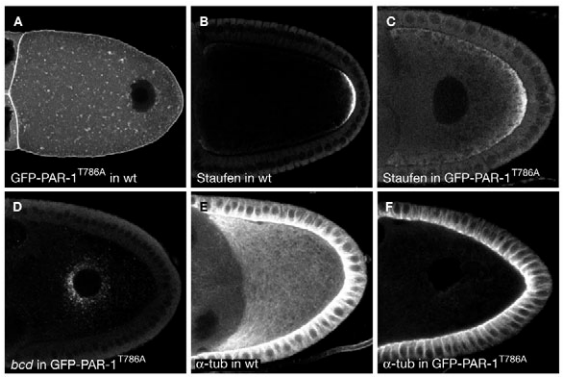

Fig. 6. aPKC phosphorylation of Par-1 is required for Par-1 localisation and oocyte polarity. (**A**) GFP-Par-1^{T786A} expression at stage 9 in a wild-type oocyte. (**B**,**C**) Staufen immunostaining at stage 9 in a wild-type oocyte (B) and in an oocyte expressing GFP-Par-1^{T786A} (C). (**D**) *bcd* mRNA in a stage 9 oocyte expressing GFP-Par-1^{T786A}. (**E**,**F**) α-Tubulin-FITC staining of a wild-type stage 9 oocyte (E) and an oocyte expressing GFP-Par-1^{T786A} (F).

Suzuki et al., 2004). To determine whether this mode of regulation is conserved in *Drosophila* Par-1, we expressed UAS:GFP-Par-1^{T786A} at mid-oogenesis. Non-phosphorylatable Par-1^{T786A} localised all around the cortex of the oocyte, indicating that Par-1 is normally excluded from the anterior and lateral cortex by aPKC phosphorylation of this site (Fig. 6A).

Expression of the non-phosphorylatable form of Par-1 disrupted oocyte polarity. The nucleus was never localised to the dorsal/anterior corner of the oocyte as in the wild type, and was instead mislocalised to the centre of the cell (Fig. 6A,C,D,F). In addition, Staufen protein and *osk* mRNA localised weakly to the posterior and spread towards the lateral cortex (Fig. 6B,C and data not shown). Like Staufen and *osk* mRNA, Kin:βgal localised more weakly to the posterior of the oocyte and in a broader crescent, suggesting that the distribution of microtubule plus ends was similarly affected (data not shown). By contrast, *bcd* mRNA was completely delocalised from the anterior cortex and formed a ring around the misplaced oocyte nucleus (Fig. 6D).

Since the localisations of *bcd* and *osk* mRNAs and the nucleus are microtubule dependent, we also examined the arrangement of the microtubules in Par-1^{T786A}-expressing oocytes by staining with an α-Tubulin antibody labelled with FITC. Par-1^{T786A} had no effect on the organisation of the microtubules prior to the repolarisation of the oocyte, with the microtubules nucleated normally from the MTOC at the posterior of the oocyte and extending through the ring canals into the nurse cells (data not shown). By contrast, non-phosphorylatable Par-1 completely disrupted the microtubule organisation at stage 9. In wild-type oocytes, most microtubules are nucleated and/or anchored at the anterior/lateral cortex of the oocyte to form an anterior-to-posterior gradient of microtubules in the cytoplasm, while a second population of microtubules appear to be nucleated from the nuclear envelope (Fig. 6E) (Januschke et al., 2006). By contrast, Par-1^{T786A}-expressing oocytes appeared to lack all cortex-associated microtubules (Fig. 6E,F). In some cases, a halo of microtubules was observed around the nucleus (data not shown).

These results suggest that the uniform cortical localisation of Par-1^{T786A} causes the whole cortex to take on the character of the wild-type posterior cortex. The nucleation or anchoring of microtubule minus ends at the anterior is suppressed, resulting in a radially symmetric oocyte in which *bcd* mRNA and microtubule minus ends localise around the nucleus in the centre of the oocyte, whereas microtubule plus ends and *osk* mRNA can localise around the entire cortex. This phenotype resembles that of *baz*$^{358-12}$ germline clones.

Overexpression of Baz$^{S151A\ S1085A}$-GFP produced the opposite phenotype to GFP-Par-1^{T768A} and a similar phenotype to strong *par-1* mutants: *bcd* mRNA was localised in ectopic patches along the lateral cortex, whereas most *osk* mRNA was localised to the centre of the oocyte, and there was a high density of microtubules throughout the oocyte, rather than the anterior-to-posterior gradient seen in the wild type (Fig. 7A-K) (Benton and St Johnston, 2003b). Thus, uniform cortical Baz induces much of the cortex to behave like the anterior cortex. The AP gradient of microtubules in wild-type oocytes and the anterior and posterior localisations of *bcd* and *osk* mRNAs therefore seem to reflect the complementary cortical distributions of Baz and Par-1 to the anterior/lateral and posterior regions of the cortex, respectively. Microtubules are nucleated or anchored from the cortical regions where the Baz complex is active, whereas this is suppressed in the posterior Par-1 domain, which is competent to anchor *osk* mRNA.

Par-1 acts downstream of Baz to control microtubule organisation in the oocyte

One important unanswered question is which of the PAR proteins organises the microtubule cytoskeleton, i.e. does the Baz complex promote microtubule nucleation/anchoring at the regions of the cortex where it is active, or does posterior Par-1 suppress the cortical nucleation/anchoring of microtubules that is otherwise constitutive? It is impossible to resolve this question using mutants in *baz* or *par-1* because the absence of either protein leads to the spreading of the other around the cortex. One way that this question can be addressed is by co-expressing the non-phosphorylatable forms of Baz and Par-

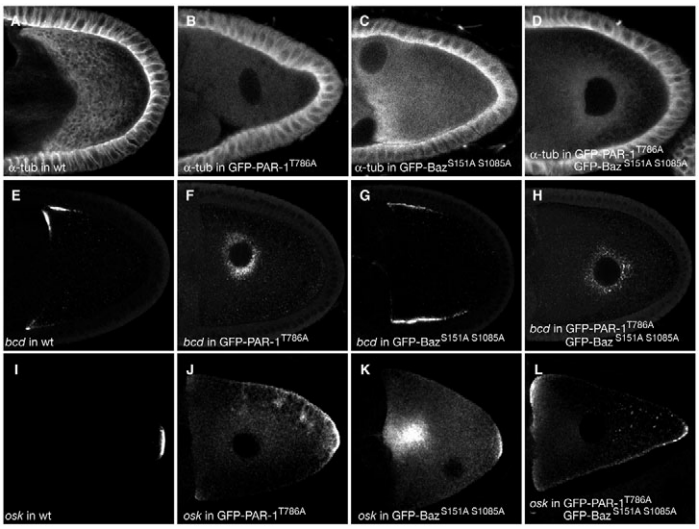

Fig. 7. Par-1 acts downstream of Baz to control microtubule organisation in the *Drosophila* oocyte. (**A-L**) α-Tubulin-FITC staining (A-D), *bcd* mRNA localisation (E-H) and *osk* mRNA localisation (I-L) in a wild-type oocyte (A,E,I), in a GFP-Par-1^{T786A}-expressing oocyte (B,F,J), in a Baz$^{S151A\ S1085A}$-GFP-expressing oocyte (C,G,K), and in an oocyte co-expressing GFP-Par-1^{T786A} and Baz$^{S151A\ S1085A}$-GFP (D,H,L) at stage 9.

1 in the same oocytes, so that both localise all around the cortex and are presumably active everywhere. When Baz$^{S151A\ S1085A}$-GFP and GFP-Par-1^{T768A} were co-expressed, the resulting oocytes showed a phenotype that is indistinguishable from that produced by expressing GFP-Par-1^{T768A} alone: cortical microtubules were completely absent and the remaining microtubules formed a halo around the misplaced oocyte nucleus (Fig. 7D). In addition, *bcd* mRNA localised around the nucleus, whereas *osk* mRNA was found all around the cortex (Fig. 7H,L). This phenotype is not caused by the downregulation or inactivation of non-phosphorylatable Baz, because Baz protein still localised around the entire cortex and recruited endogenous Par-6 (data not shown). Thus, uninhibitable Par-1 is epistatic to uninhibitable Baz, strongly suggesting that Par-1 is the major effector of microtubule organisation in the oocyte.

DISCUSSION

The *baz$^{358-12}$* allele causes a fully penetrant defect in the localisation of *bcd* and *osk* mRNAs and in the positioning of the oocyte nucleus and *gurken* mRNA, providing the first demonstration that Baz is required for the polarisation of the *Drosophila* AP and dorsal-ventral axes. This raises the question of why *baz*-null mutant germline clones that escape the block in early oogenesis sometimes develop into eggs with normal polarity. Although it is formally possible that Baz is not absolutely essential for oocyte polarity and that the *baz$^{358-12}$* allele has a dominant-negative effect, this seems very unlikely. First, *baz$^{358-12}$* behaves like a typical hypomorphic mutation as it is recessive and fails to complement the lethality of *baz*-null alleles. Second, nearly half of the escapers from *baz*-null germline clones show similar polarity defects to *baz$^{358-12}$* at stage 9, indicating that this is a loss-of-function phenotype. Thus, it seems more likely that whatever allows a few of the null germline clones to escape the early-arrest phenotype also allows some of them to escape the polarity defect at stage 9. For example, other polarity pathways might be activated in *baz*-null mutant germaria that can partially compensate for the loss of Baz in both oocyte determination and axis formation.

The observation that *baz$^{358-12}$* does not cause any defects in the initial polarisation of the oocyte, although it is essential for the AP polarisation at stage 9, indicates that there must be some differences in the functions of Baz at each stage. During early oogenesis, Baz localises in a ring around each ring canal at the anterior of the oocyte and shows perfect colocalisation with DE-cadherin (Shotgun – FlyBase) and Armadillo (Huynh et al., 2001a). Since the PDZ domains of Baz have been shown to interact with Armadillo, it might be recruited to the anterior rings through this interaction (Wei et al., 2005), which should still occur normally in the *baz$^{358-12}$* mutant. By contrast, the truncated Baz protein does not localise to the cortex of the oocyte at stages 7-9, indicating that the C-terminal region is necessary for its cortical recruitment at this stage. The only identified domain in this region is CR3, which binds to the kinase domain of aPKC. However, a point mutation in CR3 that disrupts its interaction with aPKC has no effect on the cortical localisation of Baz at stage 9 (Morais de Sa et al., 2010). There must therefore be another domain in the C-terminal region of Baz that is required for its recruitment to the oocyte cortex.

Another important difference between the initial polarisation of the oocyte and the repolarisation at mid-oogenesis is the relationship between the PAR proteins. During early oogenesis, the localisation of Baz is unchanged by loss of Par-1 and vice versa (Huynh et al., 2001a). By contrast, Baz and Par-1 show mutually exclusive localisations at stage 9, with Par-1 spreading around the lateral cortex in *baz* mutants, and Baz and Par-6 localising to the posterior in *par-1* mutants. Baz is required to recruit Par-6 to the cortex in mid-oogenesis, as Par-6 disappears from the anterior cortex in *baz$^{358-12}$* clones and localises to the posterior with Baz$^{S151A\ S1085A}$-GFP. Thus, Baz, Par-6 and presumably also aPKC form a complex in the stage 9 oocyte, making the arrangement of PAR proteins much more similar to that in the *C. elegans* zygote, with Baz (PAR-3), Par-6 and aPKC defining the anterior and lateral cortex and Par-1 the posterior. As in *C. elegans*, these complementary localisations are also maintained by mutual antagonism between the anterior and posterior PAR proteins. We have previously shown that Par-1 phosphorylates Baz to exclude it from the posterior (Benton and St Johnston, 2003b). Here we show that mutation of the conserved aPKC site in the Par-1 linker region leads to the mislocalisation of Par-1 around the anterior and lateral cortex, strongly suggesting that aPKC phosphorylates this site to restrict Par-1 to the posterior.

Although the final pattern of PAR proteins in the stage 9 *Drosophila* oocyte is similar to that in the *C. elegans* zygote, this pattern develops over a much longer period of time and in a different way. Baz-GFP is enriched at the posterior of the oocyte at the beginning of stage 7 and gradually spreads anteriorly during

the succeeding 12 hours, before finally disappearing from the posterior at stage 9. Since Par-1 appears at the posterior early in stage 7, Baz and Par-1 overlap at the posterior for some considerable time. By contrast, Par-6-Cherry starts to disappear from the posterior during stage 7, and already shows a complementary pattern to Par-1 at stage 8. This raises the question of why Par-6, which is recruited to the cortex by Baz, disappears more rapidly from the posterior. Although this might mean that they are excluded by different mechanisms, both Par-6 and Baz localise to the posterior in *par-1* mutants and in $Baz^{S151A\ S1085A}$-GFP-expressing oocytes, indicating that their exclusion depends on the phosphorylation of Baz by Par-1. Thus, Par-1 phosphorylation might first release Par-6 from Baz, and then more gradually displace Baz from the cortex. The phosphorylation of serine 1085 of Baz by Par-1 disrupts the interaction of Baz with aPKC and this might be sufficient to release the Par-6–aPKC complex. However, Par-6 also binds directly to the PDZ domains of Baz, and the phosphorylation of serine 1085 alone would not be expected to interfere with this interaction. Thus, Par-1 might also act in some other way to release Par-6, perhaps by promoting the posterior recruitment of Lgl, as the latter is known to inhibit the interaction of Par-6–aPKC with Baz in neuroblasts (Wirtz-Peitz et al., 2008).

The gradual evolution of PAR protein localisation during stages 7-9 argues against the idea that the oocyte is polarised by a cortical contraction, as in *C. elegans*, and we have never observed any evidence for cortical movements of the actin cytoskeleton. This raises the question of how this asymmetry arises. We can envisage two possible scenarios for how the polarising signal from the posterior follicle cells triggers PAR protein asymmetry. First, the initial cue could remove or inactivate aPKC and Par-6 at the posterior, which would then allow Par-1 to localise there because aPKC is no longer present or able to exclude it. Although aPKC can be inhibited at the posterior by Lgl, this seems unlikely to provide the cue because Lgl localises to the posterior after Par-1 and is not essential for oocyte polarity. Alternatively, the initial asymmetry could be generated by the posterior recruitment and activation of Par-1. Work in mammals has shown that LKB1 (STK11) phosphorylates the activation loop of PAR-1 (MARK2) to turn on its kinase activity (Lizcano et al., 2004), and this is likely to be case in *Drosophila* as well, as *lkb1* mutants exhibit a very similar phenotype to *par-1* mutants (Martin and St Johnston, 2003). LKB1 activity is regulated by protein kinase A (PKA), which is required for the transduction of the polarising follicle cell signal in the oocyte (Lane and Kalderon, 1994; Martin and St Johnston, 2003). Thus, it is possible that the initial asymmetry is generated by a kinase cascade at the posterior of the oocyte, consisting of PKA, which activates LKB1, which activates Par-1.

Once the PAR polarity has been established, it must somehow polarise the oocyte microtubule cytoskeleton to direct the localisation of *bcd* and *osk* mRNAs. Our epistasis experiment suggests that Par-1 provides the primary output from the PAR system, as uniformly distributed Par-1 makes the whole cortex behave like the posterior cortex regardless of whether Baz is also uniformly distributed or not. Based on the *par-1* loss- and gain-of-function phenotypes in the oocyte and follicle cells, Par-1 might act to stabilise microtubule plus ends at the cortex and to inhibit the nucleation or anchoring of microtubule minus ends.

One key remaining question is the identity of the Par-1 substrates that mediate its effect on microtubule organisation. In addition to Baz, Par-1 has also been shown to phosphorylate Exuperantia and Ensconsin in the oocyte to regulate *bcd* mRNA localisation and the activity of Kinesin (Riechmann and Ephrussi, 2004; Sung et al., 2008). However, neither of these targets can account for the dramatic effects of Par-1 on microtubule organisation. It has recently been claimed that Par-1 regulates the oocyte microtubule cytoskeleton by phosphorylating the microtubule-stabilising protein Tau, thereby destabilising the microtubules at the posterior of the oocyte (Tian and Deng, 2009). This conclusion was based on the observation that germline clones of $tau^{Df(3R)MR22}$ produce a partially penetrant defect in the anchoring of the oocyte nucleus. However, the $tau^{Df(3R)MR22}$ mutation is a 65 kb deletion that removes eight other genes as well as *tau*, and the phenotype could therefore be due to the loss of one of these other loci. More importantly, *tau* can be specifically removed without deleting any other genes by generating heterozygotes for two overlapping deficiencies, and these *tau*-null flies are homozygous viable and fertile and develop normally polarised oocytes (Doerflinger et al., 2003). Thus, it seems highly unlikely that Tau is a relevant substrate for Par-1 in the polarisation of the oocyte. A full understanding of oocyte polarity will therefore depend on the identification of the Par-1 targets that control microtubule nucleation, anchoring and stability.

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Competing interests statement

The authors declare no competing financial interests.

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