The RNA-binding protein Squid is required for the establishment of anteroposterior polarity in the *Drosophila* oocyte

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Summary

The heterogeneous nuclear ribonucleoprotein (hnRNP) Squid (Sqd) is a highly abundant protein that is expected to bind most cellular RNAs. Nonetheless, Sqd plays a very specific developmental role in dorsoventral (DV) axis formation during *Drosophila* oogenesis by localizing *gurken* (*grk*) RNA. Here, we report that Sqd is also essential for anteroposterior (AP) axis formation. We identified *sqd* in a screen for modifiers of the *Protein Kinase A* (*PKA*) oogenesis polarity phenotype. The AP defects of *sqd* mutant oocytes resemble those of *PKA* mutants in several ways. In both cases, the cytoskeletal reorganization at mid-oogenesis, which depends on a signal from the posterior follicle cells, does not produce a correctly polarized microtubule (MT) network. This causes the posterior determinant, *oskar* (*osk*) RNA, to localize to central regions of the oocyte, where it is ectopically translated. Additionally, MT-dependent anterior movement of the oocyte nucleus and the *grk*-dependent specification of posterior follicle cells are unaffected in both mutants. However, in contrast to *PKA* mutants, *sqd* mutants do not retain a discrete posterior MT organizing center (MTOC) capable of supporting ectopic posterior localization of *bicoid* (*bcd*) RNA. *sqd* mutants also display several other phenotypes not seen in *PKA* mutants; these probably result from the disruption of MT polarity in earlier stages of oogenesis. Loss of Sqd does not affect polarity in follicle cells, wings or eyes, indicating a specific role in the determination of MT polarity within the germline.

Key words: *Drosophila*, Oogenesis, Polarity, Microtubules, Squid, PKA, hnRNP

Introduction

Many cells have an obvious polarity that is essential to their function. One important mechanism for establishing polarity involves the asymmetric localization of mRNAs, which is then translated into asymmetric protein localization and function. In many cases, RNAs are localized by directed transport along microtubules (MTs) (Lipshitz and Smibert, 2000). This is true of the *Drosophila* oocyte, in which the MT-dependent localization of three primary mRNA determinants polarizes the cell and establishes the body plan of the future embryo. Localization of *bicoid* (*bcd*) and *oskar* (*osk*) mRNAs to the anterior and posterior termini of the oocyte, respectively, establishes the anteroposterior (AP) axis, and localization of *gurken* (*grk*) mRNA to the presumptive dorsal anterior corner determines the dorsoventral (DV) axis (van Eeden and St Johnston, 1999). The large size of the *Drosophila* oocyte, along with its relatively slow development, has allowed substantial dissection of the processes involved in the establishment of polarity using molecular genetics, thus affording insights into fundamental mechanisms that organize MT polarity and localize RNAs (Johnstone and Lasko, 2001; Riechmann and Ephrussi, 2001).

The *Drosophila* oocyte develops within an egg chamber, which consists of 15 nurse cells and an oocyte surrounded by an epithelial monolayer of somatic follicle cells (Spradling, 1993). The oocyte and nurse cells are produced from a single germline cystoblast, which arises when a germline stem cell divides. The cystoblast undergoes four synchronous mitotic divisions to produce a germline cyst of 16 cystocytes; each division ends with incomplete cytokinesis, leaving the cystocytes interconnected by cytoplasmic bridges called ring canals. As the cyst forms, MTs are organized by a specialized cytoskeletal structure called the fusome, which spans the entire cyst. During the cystocyte divisions, the fusome attaches to one pole of every mitotic spindle, ensuring the stereotypical arrangement of cystocytes (Grieder et al., 2000). Subsequently, the fusome directs the formation of an MT organizing center (MTOC) within the oocyte, which enables cytoplasmic determinants to accumulate within the ooplasm and maintain oocyte fate (Huynh and St Johnston, 2004). The cyst then rearranges such that the oocyte is positioned posteriorly, and somatic follicle cells encapsulate the cyst. At this stage, defined as stage 1, the MTOC is positioned at the anterior of the oocyte, and the MT plus-ends extend through the ring canals into the nurse cells (Theurkauf et al., 1992). At stage 2, the egg chamber buds off from the germarium, where it is formed, and enters the vitellarium, where it will develop into a mature egg. The MTOC and the oocyte nucleus move to the posterior of the oocyte (Theurkauf et al., 1992), and the polarized MT network directs the localization of several cytoplasmic factors that are synthesized in the nurse cells to the oocyte (Koch and Spitzer, 1983; Pokrywka and Stephenson, 1995). In this

manner *grk* RNA becomes concentrated within the oocyte and accumulates at its posterior cortex, where it is translated during stages 2-6 (Neuman-Silberberg and Schupbach, 1993; Van Buskirk and Schupbach, 1999). The posteriorly localized Grk protein, a TGF-α homolog, signals via the Epidermal growth factor receptor (EGFR) to the follicle cells overlying the oocyte at the posterior of the egg chamber, causing posterior cells to express different markers and behave differently from the other follicle cells (Gonzalez-Reyes et al., 1995; Gonzalez-Reyes and St Johnston, 1998; Roth et al., 1995). At stage 7-8, there is a rearrangement of the germline MT cytoskeleton such that the MT network emanating from the posterior MTOC is replaced by MTs nucleated from multiple sites at the anterior cortex of the oocyte and extending toward the posterior (Theurkauf et al., 1992). The newly polarized MT cytoskeleton allows *bcd* RNA to be directed toward the anterior of the oocyte in association with a minus-end directed motor (Arn et al., 2003; Schnorrer et al., 2000), and *osk* RNA to be transported to the posterior by the plus-end directed motor, kinesin (Brendza et al., 2000). MT rearrangement also allows the oocyte nucleus to move from its early position near the posterior to the presumptive dorsal anterior corner (Koch and Spitzer, 1983; Swan et al., 1999). During stages 8-11, *grk* RNA accumulates in a cap between the nucleus and the plasma membrane, and the resulting Grk protein signals via the EGFR to specify dorsal cell fate in the overlying follicular epithelium (Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1996).

Mutations that alter Grk signaling during stages 8-11 disrupt DV patterning of the follicle cells, leading to altered DV polarity in the eggshell and embryo. For instance, loss of Squid (Sqd) activity in the germline leads to the delocalization of *grk* RNA and Grk protein along the entire anterior circumference of the oocyte, resulting in loss of ventral follicle cell fates (Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1996). Mutations that prevent Grk signaling to the posterior follicle cells during stages 2-6 disrupt AP patterning of the oocyte. For example, mutations in *grk* itself lead to an aberrant MT rearrangement at stages 7-8, altered localization of *bcd* and *osk* RNAs, and failure of the oocyte nucleus to migrate to the anterior (Gonzalez-Reyes et al., 1995; Roth et al., 1995). This, along with the discovery of similar AP phenotypes when specific gene activities (e.g. Notch and Laminin A) are lost in the follicle cells alone (Deng and Ruohola-Baker, 2000; Ruohola et al., 1991), led to the proposal that correctly specified posterior follicle cells must signal to the oocyte at stages 6-7 to allow normal MT rearrangement. However, this hypothetical signal has not been defined.

Protein kinase A (PKA; PKA-C1 – FlyBase) was identified as a potential transducer of the posterior follicle cell signal because it is required in the germline for normal MT polarity and RNA localization in stage 8-9 oocytes (Lane and Kalderon, 1994). In this study, we characterized the role of PKA further and identified a mutation in *sqd* as a weak modifier of the *PKA* mutant phenotype. Sqd is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) A/B family of RNA-binding proteins, and its role in establishing DV polarity has been well characterized (Kelley, 1993; Matunis et al., 1994; Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1996). We found that loss of Sqd function produces a highly penetrant defect in MT organization along the AP axis at stages 8-9, as well as aberrant localization of *osk* RNA and other molecules that are normally localized posteriorly. A less penetrant defect in MT polarity, which affects Grk localization, is also evident before stage 6 in *sqd* mutants. Despite this earlier defect, *sqd* mutants display normal specification of posterior follicle cells in response to the early Grk signal. Thus, Sqd is essential for the establishment of MT polarity in both early and mid-oogenesis.

Materials and methods

Drosophila stocks

Mutant clones were generated by mitotic recombination using the FLP/FRT system and an X-chromosomal *hs-flp* (Xu and Rubin, 1993). Germline clones were made using the DFS technique (Chou et al., 1993; Chou and Perrimon, 1996) and the following alleles: $PKA\text{-}C1^{H2}$ (Lane and Kalderon, 1993), P-element lethal mutations from BDGP (Spradling et al., 1999), sqd^{ix50} and sqd^{ix77} (gifts from T. Schupbach). Other alleles used for genetic analyses were: $PKA\text{-}C1^{A13}$ and *Df(2L)Tw2* (Lane and Kalderon, 1993), sqd^{1} and *Df(3R)urd* (gifts from A. Norvell), $hrp48^{k16203}$ (gift from A. Ephrussi), $hrp48^{K02814}$, $hrp48^{02647}$, *Df(3R)126C*, $mago^{1}$ and *Df(2R)F36* (from Bloomington Stock Center). Somatic clones were marked using *FRT82B Ubi-GFP* (from Bloomington) or *FRT82B hsCD2,* y^{+} (gift from G. Struhl). Rescue constructs used were: mC* (Li et al., 1995), GFP-$LKB1^{S535E}$ (Martin and St Johnston, 2003) and SqdS (Norvell et al., 1999). Marker lines used were: $mirror^{6D1}$ (Ruohola-Baker et al., 1993), *Orb-Pz* (Lantz and Schedl, 1994), $pnt^{998/12}$, *BB127*, *L53b* and *5A7* (Gonzalez-Reyes and St Johnston, 1998), *kinesin-lacZ*, pnt^{rm254} (Lee and Montell, 1997), *GFP-Stau* (gift from D. St Johnston) and $m1^{414}lacwt$ (Gunkel et al., 1998).

RNA in-situ hybridization

Ovaries were hand-dissected from ten females fattened on yeast paste. Ovaries were teased open with forceps and fixed for 9 minutes in a biphasic 1:6 mixture of 6% formaldehyde:heptane. Ovaries were washed in PBS + 0.1% Tween-20 (PBT), permeabilized for 1 hour in PBT + 1% Triton-X 100, dehydrated into methanol, and stored overnight at –20°C in 100% methanol. After rehydration, ovaries were treated for 10 minutes with 50 μg/ml proteinase K and for 2 minutes with 2 mg/ml glycine. Ovaries were post-fixed for 15 minutes in 4% formaldehyde and washed 5×5 minutes in PBT. Subsequent steps were according to Tautz and Pfeifle with slight adjustments. Digoxigenin-labeled riboprobes were hybridized at 1:50 overnight at 55°C. Hybridization buffer was adjusted to pH 5. Alkaline-phosphatase-conjugated anti-digoxigenin antibody was used at 1:2000 (Roche). Ovaries were mounted in 60% glycerol in PBS.

Riboprobes were synthesized from cDNAs subcloned into a Bluescript vector (*bcd*) or a pNB40 vector (*osk*) using Ampliscribe transcription kit (Epicentre) and DIG RNA Labeling Mix (Roche). Following the transcription reaction, probes were hydrolyzed in carbonate buffer (2×: 120 mmol/l Na_2CO_3, 80 mmol/l $NaHCO_3$, pH 10.2) for 20 minutes at 65°C. Hydrolysis was stopped with one volume 0.2 mol/l NaOAc (pH 6), and the probes were precipitated at –20°C with 0.1 volume 4 mol/l LiCl, 20 μg/ml tRNA, and 2.5 volumes chilled 100% ethanol. Probes were stored at –20°C in 200 μl hybridization buffer.

Antibody staining

Ovaries were dissected and fixed as for RNA in-situ hybridization. After washing in PBT, ovaries were blocked for 1 hour in PBT + 1% Triton X-100, 1% BSA. Primary antibodies were diluted in PBT + 0.1% BSA and incubated overnight at 4°C. Anti-Grk (mouse, 1:30), anti-Orb (mouse, 1:30) and anti-N^{intra} (mouse, 1:1000) were from the Developmental Studies Hybridoma Bank. Anti-β-galactosidase

antibodies were from Promega (mouse, 1:1000) and Cappel (rabbit, 1:4000). Anti-PKCζ was from Santa Cruz Biotech (rabbit, 1:1000). Anti-Dhc (mouse, 1:100), anti-Baz (rabbit, 1:1000), anti-Sqd (mouse, 1:10) and anti-Osk (rabbit, 1:3000) were gifts from T. Hays, A. Wodarz, T. Schupbach and A. Ephrussi respectively.

Ovaries were washed 3×10 minutes in PBT + 0.1% BSA and incubated for 1 hour at room temperature with Alexa fluor-conjugated secondary antibodies at 1:500 (Molecular Probes). After 3×10-minute washes in PBT, ovaries were mounted in Aquapolymount (Polyscience) or Fluoromount G (Southern Biotech). Before mounting, ovaries were sometimes incubated for 15 minutes in 1 mg/ml Rnase A at 37°C and stained for 20 minutes with 10 μg/ml propidium iodide (Molecular Probes) in PBT. Alternatively, ovaries were stained for 20 minutes with rhodamine-conjugated phalloidin (Molecular probes) at 1:40. β-Galactosidase activity staining was performed as described previously.

Tubulin staining

Ovaries were dissected as for RNA in-situ hybridization and fixed for 10 minutes in 100% methanol at –20°C. After rehydration into PBT, ovaries were blocked as for antibody staining. FITC-conjugated anti-α-tubulin antibody (Sigma) was incubated at 1:250 in PBT + 0.1% BSA overnight at 4°C. Ovaries were washed 3×10 minutes in PBT + 0.1% BSA, incubated for 1 hour at room temperature with Alexa 488-conjugated secondary antibodies at 1:500 (Molecular Probes), and mounted in Aquapolymount or Fluoromount G. For partial MT depolymerization, ovaries were rocked at room temperature in 20 μg/ml colchicine + 0.1% DMSO in modified Robb's medium (Theurkauf et al., 1992) for 10 minutes and rinsed in PBT before methanol fixation.

Results

Loss of PKA in the germline does not impair specification of posterior follicle cell fate

When the activity of the *Drosophila* PKA catalytic subunit, PKA-C1 (also known as DC0), is reduced in oocytes, the MT cytoskeleton fails to reorganize properly at mid-oogenesis, leading to mislocalization of *bcd* and *osk* mRNAs (Lane and Kalderon, 1994). Movement of the oocyte nucleus from the posterior to the anterior cortex was not impaired, however (Fig. 1A), despite the fact that nuclear movement is dependent on MTs and is disrupted by several other mutations that affect oocyte MT polarity (Gonzalez-Reyes et al., 1995; Koch and Spitzer, 1983; Roth et al., 1995; Swan et al., 1999). Consequently, dorsal follicle cell fate, as visualized with the *lacZ* enhancer trap *mirror*^{6D1}, is specified correctly in *PKA* mutants (Fig. 1B).

Germline clones of a *PKA-C1* null allele, *H2*, showed over 90% penetrance of mid-oogenesis AP polarity defects, as detected by a kinesin-β-galactosidase (kin-β-gal) protein that marks the plus-ends of MTs and a GFP-Staufen (GFP-Stau) (St Johnston et al., 1991) protein that generally co-localizes with *osk* RNA (Fig. 1E,G). Such polarity defects can be a consequence of failed posterior follicle cell specification, as in *grk* and *cornichon* (*cni*) mutants, for example (Gonzalez-Reyes et al., 1995; Roth et al., 1995). However, the expression of the posterior follicle cell marker *pointed*$^{998/12}$ (*pnt*) was normal in 89% of egg chambers containing *PKA-C1*^{H2} germline clones (*n*=187; Fig. 1C). *pnt*$^{998/12}$ expression was absent from posteriorly positioned follicle cells only when those cells also lacked PKA activity, consistent with previously noted cell-autonomous effects. Similarly, expression of anterior markers in posterior follicle cells surrounding *PKA* mutant germline clones was never observed for the centripetal cell marker

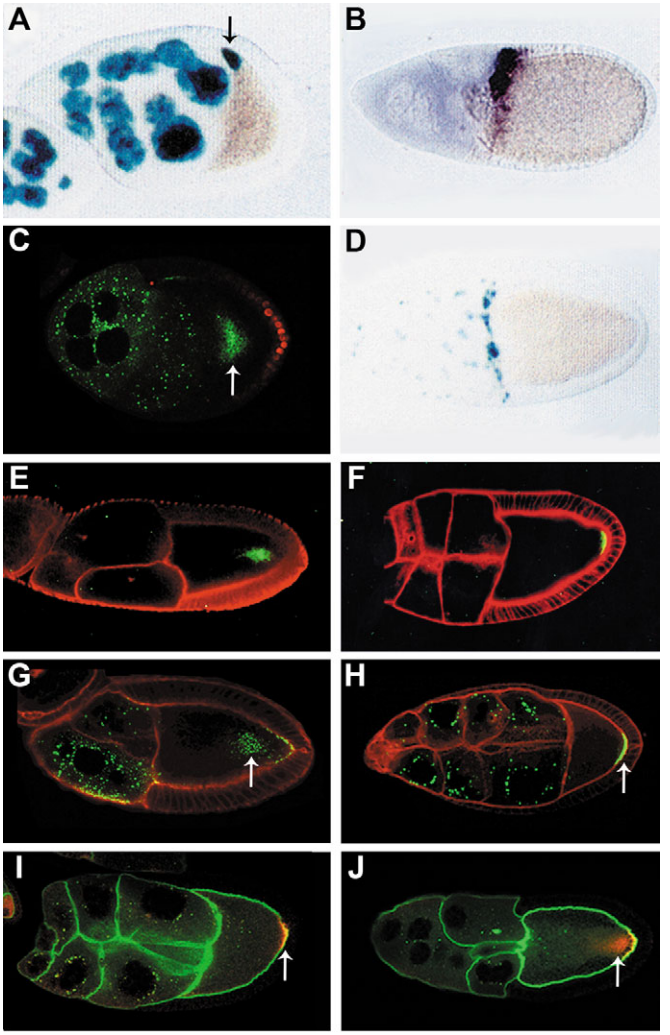

Fig. 1. *PKA* mutant phenotypes and attempted rescue by constitutive PKA activity or LKB1. (A-D) *PKA-C1* null germline clones do not prevent normal anterior migration of the oocyte nucleus (A, arrow), marked by *orb-PZ* expression in the germline nuclei (blue), normal expression of the dorsal follicle cell marker *mirror*^{6D1} (B, purple), normal expression of the posterior follicle cell marker *pnt*$^{998/12}$ (C, red; green shows GFP-Stau mislocalized to the center of the oocyte, arrow) or normal expression of the centripetal follicle cell marker *BB127* (D, blue). (E,F) Mislocalization of kin-β-gal (green) and fusion of nurse cells, revealed by rhodamine-phalloidin staining of actin filaments (red), in *PKA-C1* null germline clones (E). Both phenotypes are rescued by expression of the constitutively active mouse PKA catalytic subunit mC* from an *actin* promoter (F). (G,H) GFP-Stau (green), like kin-β-gal, is mislocalized toward the center of the oocyte in *PKA-C1* null germline clones (G, arrow) and is restored to a normal posterior localization by expression of mC* (H, arrow). Rhodamine-phalloidin (red) marks cell outlines. (I,J) Expression of the GFP-LKB1^{S535E} transgene (green) carrying a mutation that mimics phosphorylation at the putative PKA site does not perturb kin-β-gal localization (red) in wild-type oocytes (I, arrow) but fails to rescue either kin-β-gal mislocalization (red, arrow) or nurse cell fusions (outlined by GFP-LKB1^{S535E} in green) in *PKA-C1* null germline clones (J).

BB127 (Fig. 1D) and only very rarely for the general anterior marker *L53b* and the border cell marker *5A7* (5% each, data not shown). The rare ectopic expression of *L53b* and *5A7* at the posterior is probably due to loss of PKA in the follicle cells and cannot plausibly be responsible for the >90% incidence of AP polarity phenotypes in *PKA* null germline clones. Thus, loss of PKA in the germline disrupted oocyte polarity without substantially affecting established markers of posterior follicle cell identity. These results support the hypothesis that PKA affects the oocyte MT network in mid-oogenesis in response to a normal posterior follicle cell signal.

Rescue of *PKA* mutant phenotypes by a constitutively active PKA catalytic subunit

As PKA activity is essential for normal oocyte polarity, it has been suggested that PKA might actively transduce the polarizing signal from the posterior follicle cells to the oocyte (Lane and Kalderon, 1994). Therefore, we tested whether normal regulation of PKA activity is required in this setting. The PKA holoenzyme (R_2C_2) is activated by binding of cAMP to its regulatory (R) subunits, thereby releasing active monomeric catalytic (C) subunits. A specific altered mouse PKA catalytic subunit, mC*, binds poorly to regulatory subunits and is therefore constitutively active and largely unaffected by cAMP concentration or other inputs mediated by the regulatory subunits (Li et al., 1995). Expression of mC* from an *actin5C* gene promoter (by flp-mediated conversion of the conditional *actp>y$^+$>mC** transgene to *actp>mC**) rescued both the kin-β-gal and GFP-Stau mislocalization defects of *PKA-C1^{H2}* germline clones from >90% to approximately 10% penetrance (Fig. 1E-H). The fusion of nurse cell membranes, another characteristic of *PKA-C1* germline clones, was also fully rescued. Thus, PKA activity need not be regulated by cAMP or the regulatory subunits in order to promote normal oocyte polarity.

Even though mC* efficiently rescued oocyte polarity and nurse cell fusion defects in *PKA-C1^{H2}* germline clones, those eggs did not develop normally. Fewer than 4% hatched even if zygotically rescued by a wild-type paternal *PKA-C1* allele, and most were either unfertilized or arrested development very early (see Table S1A in the supplementary material). Of the embryos that hatched, roughly half developed to adulthood if zygotically rescued, but all died before third instar in the absence of zygotic rescue. mC* was also unable to rescue animals zygotically null for *PKA-C1* (but derived from heterozygous parents) beyond second instar (see Table S1B in the supplementary material). Thus, there are PKA functions in oogenesis or early embryogenesis, as well as during larval development, that are not adequately substituted by mC*.

LKB-1 does not rescue *PKA* mutant oocytes

Drosophila homologs of the *Caenorhabditis elegans partition defective* (*par*) genes are involved in organizing the germline MT cytoskeleton at mid-oogenesis (Pellettieri and Seydoux, 2002). Germline mutation of the *Drosophila par-4* homolog, *lkb1*, can produce a mid-oogenesis phenotype similar to that of *PKA* mutants. As PKA can phosphorylate LKB1 at a specific site in vitro, and as rescue activity of an LKB1 transgene is reduced if the PKA site is substituted with an alanine (GFP-LKB1^{S535A}) and enhanced if substituted with a glutamate residue (GFP-LKB1^{S535E}), it has been suggested that LKB1 is a key target of PKA in oocyte polarity development (Martin and St Johnston, 2003). If the sole role of PKA in AP polarity were to phosphorylate LKB1, then expressing the LKB1 transgene carrying the phosphorylation-mimicking mutation in the PKA site should bypass the need for PKA. However, *PKA-C1^{H2}* germline clones that overexpress GFP-LKB1^{S535E} displayed 100% kin-β-gal mislocalization (n=19; Fig. 1J), whereas wild-type oocytes overexpressing the transgene did

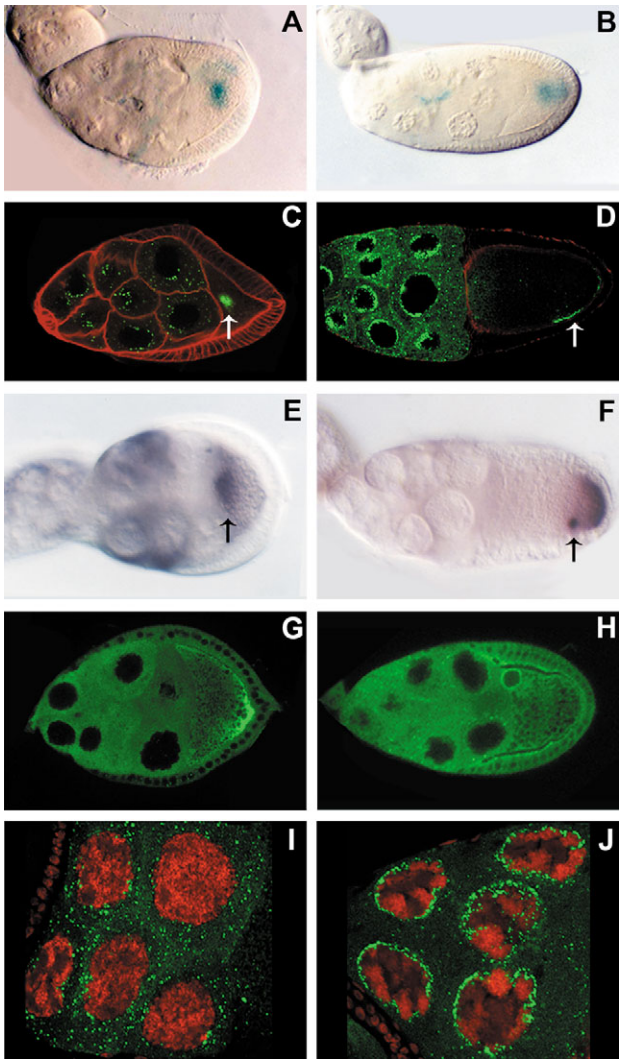

Fig. 2. *sqd* mutants display AP patterning defects at mid-oogenesis. (A,C,E) In *sqd^{j4B4}* germline clones, kin-β-gal (A, blue), GFP-Stau (C, green) and *osk* RNA (E, purple) are mislocalized to an ectopic spot in the center of the oocyte at almost complete penetrance. (B,D,F) Weaker mislocalization phenotypes are observed for kin-β-gal (B, blue), GFP-Stau (D, green) and *osk* RNA (F, purple) in *sqd^{j4B4}/sqd^1* (B,F) and *sqd^1/sqd^1* (D) egg chambers. Arrows point to ectopic GFP-Stau (C,D) and *osk* RNA (E,F). Rhodamine-phalloidin (red) marks cell outlines in C and D. (G,H) Dynein heavy chain (green) localizes to the posterior in wild-type stage 9-10 oocytes (G) but not in *sqd^{j4B4}* germline clone egg chambers (H). (I,J) GFP-Stau (green) appears as diffuse particles in the nurse cell cytoplasm of wild-type egg chambers (I), while in *sqd^{j4B4}* germline clones, it aggregates around the nurse cell nuclei (J). Nuclei are labeled with propidium iodide (red). Note that *sqd^{j4B4}* germline clone oocytes are often smaller than wild-type oocytes in mid- to late oogenesis.

not exhibit any AP polarity defects (Fig. 1I). Thus, LKB1 does not seem to be a major target for PKA function in oocyte polarity.

A P-element insertion in *sqd* causes an AP patterning defect in the oocyte

In an effort to identify factors involved in transducing the mid-oogenesis signal with PKA, we performed a dominant enhancer screen in a *PKA* hypomorphic background. We found that the P-element allele *l(3)j4B4* dominantly enhanced the kin-β-gal mislocalization phenotype of the *PKA* hypomorph from less than 10% penetrance to 29%. In the absence of any *PKA* mutation, *l(3)j4B4* homozygous germline clones made from either of two independent recombinant chromosomes showed over 90% kin-β-gal mislocalization in stage 9 (n=58; Fig. 2A) and close to 100% GFP-Stau mislocalization in stages 9 and 10 (n=106; Fig. 2C). Like *PKA* germline clones, *l(3)j4B4* germline clones showed no defect in movement of the oocyte nucleus to the anterior cortex (data not shown). To verify that the P-element insertion was the cause of the phenotype, we generated a revertant line in which the P-element had been mobilized and excised completely out of the genome. The revertant line was homozygous viable and showed 93% normal GFP-Stau localization (n=149; data not shown), a penetrance that is well within the range of GFP-Stau localization observed for various controls. The *l(3)j4B4* P-element is inserted into the first intron of the *sqd* gene (http://flybase.org/). Thus, a P-element insertion in *sqd* is responsible for a strong defect in AP patterning in the oocyte.

sqd alleles cause AP and DV patterning defects

To test whether *l(3)j4B4* behaves like other alleles of *sqd*, we examined *l(3)j4B4* germline clones for classic *sqd* phenotypes. DV defects, including eggshells with dorsal appendage material around their entire anterior circumference, specification of dorsal fate in all the follicle cells surrounding the anterior of the oocyte, and Grk protein accumulation along the entire anterior cortex of stage 9-10 oocytes, were observed for *l(3)j4B4* germline clones (see Fig. S1A-D in the supplementary material). Furthermore, in both *l(3)j4B4* germline clones and sqd^{1} mutant ovaries, the nurse cell chromosomes failed to disperse completely beyond stage 6 (see Fig. S1F in the supplementary material) (Goodrich et al., 2004), in contrast to wild-type oocytes, in which the nurse cell chromosomes remain polytene only for the early stages of oogenesis (see Fig. S1E in the supplementary material, arrow). Finally, no Sqd protein was detected in the nurse cells or oocyte in *l(3)j4B4* germline clones (see Fig. S1H in the supplementary material). Thus, *l(3)j4B4* mutants clearly are deficient for Sqd activity, displaying all the previously described oogenesis phenotypes characteristic of *sqd* mutants. We conclude that *l(3)j4B4* is indeed an allele of *sqd*, and we now refer to it as sqd^{j4B4}.

Because sqd^{j4B4} shows a highly penetrant defect in AP patterning during oogenesis, we examined whether other *sqd* alleles also exhibit a similar phenotype. We found GFP-Stau to be substantially mislocalized in stage 9-10 oocytes of escaper females from all strong *sqd* allelic combinations tested, including sqd^{j4B4}/sqd^{ix50}, sqd^{j4B4}/sqd^{ix77}, sqd^{j4B4}/Df, sqd^{ix77}/Df, as well as in females containing sqd^{ix77} germline clones (Table 1A). For all these genotypes, we often observed a small amount of GFP-Stau in the correct location at the posterior of the oocyte, in addition to the ectopic GFP-Stau in the center of the oocyte, and the incidence of oocytes with normal localization was consistently higher in later stages. We also observed GFP-Stau mislocalization in homozygotes of the weak, viable allele sqd^{1} and in sqd^{j4B4}/sqd^{1} trans-heterozygotes (Table 1A). In these genotypes, GFP-Stau rarely accumulated as a 'cloud' in the center of the oocyte as it does in sqd^{j4B4} germline clones; rather it seemed to aggregate in 'flecks' in the ooplasm or in ectopic locations along the lateral cortex (Fig. 2D). Additionally, all *sqd* alleles showed aberrant GFP-Stau aggregation surrounding the nuclei of the nurse cells (Fig. 2J). A SqdS transgene that expresses one of the three described Sqd splice variants (Norvell et al., 1999) rescued the partial lethality of sqd^{j4B4} in combination with other strong *sqd* alleles and also restored normal GFP-Stau localization in those females (Table 1A,B). Both the lethality complementation tests and the GFP-Stau mislocalization data suggest that sqd^{j4B4} is a stronger allele than the previously described allele sqd^{ix77}, which is the result of a small deletion in the 5′ UTR (Kelley, 1993) (Table 1). Germline clones of the probable null allele, sqd^{ix50} (Kelley, 1993), did not produce vitellogenic egg chambers. We conclude from these data that all *sqd* alleles disrupt AP polarity during oogenesis to a degree commensurate with their allelic strength.

Table 1. A Sqd transgene rescues AP polarity and viability of *sqd* mutants

A

Genotype	% Mislocalization of GFP-Staufen	n
sqd^{j4B4}/sqd^{j4B4} glcs	99	106
sqd^{j4B4}/sqd^{ix50}	93	74
sqd^{j4B4}/sqd^{ix77}	88	86
sqd^{j4B4}/sqd^{1}	65	138
sqd^{1}/sqd^{1}	43	998
$sqd^{j4B4}/Df(3R)urd$	81	53
$sqd^{ix77}/Df(3R)urd$	99	92
sqd^{ix77}/sqd^{ix77} glcs	58	42
$P[sqdS]/+;\ sqd^{j4B4}/sqd^{j4B4}$ glcs	18	73
$P[sqdS]/+;\ sqd^{j4B4}/sqd^{ix50}$	6	361
$P[sqdS]/+;\ sqd^{j4B4}/sqd^{ix77}$	22	318

B

Genotype	% Viability*	n
sqd^{j4B4}/sqd^{ix77}	33	250
sqd^{j4B4}/sqd^{ix50}	26	203
$sqd^{j4B4}/Df(3R)126C$	12	192
$sqd^{j4B4}/Df(3R)urd$	8	492
$sqd^{ix77}/Df(3R)urd$	37	96
$sqd^{ix50}/Df(3R)urd$	0	204
$P[sqdS]/+;\ sqd^{j4B4}/sqd^{ix77}$	83	232
$P[sqdS]/+;\ sqd^{j4B4}/sqd^{ix50}$	120	232
$P[sqdS]/+;\ sqd^{j4B4}/Df(3R)126C$	101	152
$P[sqdS]/+;\ sqd^{j4B4}/Df(3R)urd$	85	37

glcs, germline clones.
*Number of flies of each genotype divided by the expected number of flies for that genotype ×100. Expected numbers were calculated based on the numbers of balancer siblings.

osk RNA is mislocalized in mid-oogenesis *sqd* mutants as a result of altered MT polarity

In order to characterize further the effect of Sqd on AP

patterning, we examined key molecules that normally localize to the posterior of oocytes at stage 9 and later. *osk* RNA was mislocalized in 95% of sqd^{j4B4} germline clones (n=61), usually to the center of the oocyte (Fig. 2E). A less penetrant transient mislocalization of *osk* RNA at the anterior of stage 9 sqd^{1} oocytes was recently reported (Norvell et al., 2005). We observed some anterior *osk* RNA in a small percentage of sqd^{j4B4} germline clones but not in sqd^{j4B4}/sqd^{1} oocytes. In sqd^{j4B4}/sqd^{1} oocytes, *osk* RNA was mislocalized at high penetrance (74%, n=163), typically appearing as 'flecks' in the ooplasm or along the lateral cortex in a pattern similar to that of mislocalized GFP-Stau in oocytes of this genotype (Fig. 2F).

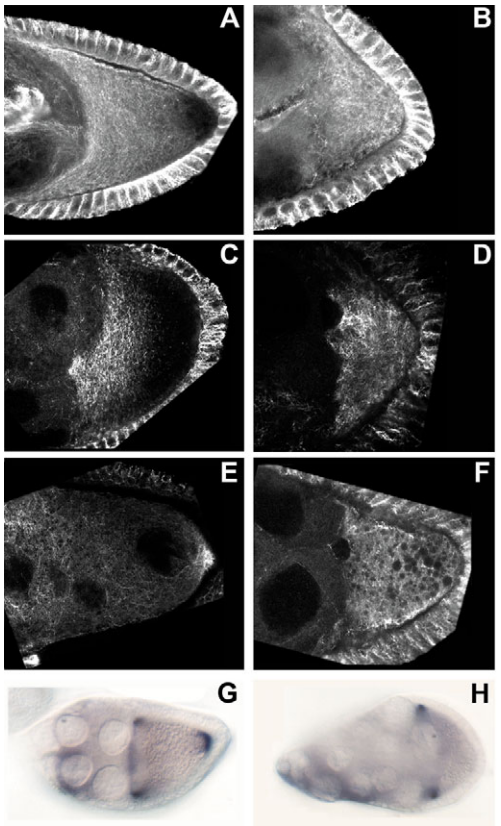

Fig. 3. MT distribution is altered in sqd^{j4B4} germline clones at mid-oogenesis, but *bcd* localization is normal. (A,B) FITC-conjugated α-tubulin antibody staining of stage 9 wild-type oocytes (A) shows a gradient of MTs extending from the anterior cortex to the posterior, with the posterior being relatively free of tubulin staining. By contrast, stage 9 sqd^{j4B4} germline clone oocytes (B) display an even distribution of tubulin staining along the entire oocyte cortex. (C-F) α-tubulin staining following partial MT depolymerization reveals short MT stubs emanating from the anterior cortex of stage 9 wild-type oocytes (C), whereas in sqd^{j4B4} germline clone oocytes, such stubs are visible along the entire oocyte cortex (D,F). The oocyte in F is slightly older than that shown in D. In $PKA\text{-}C1^{H2}$ germline clone oocytes, MT stubs are visible along the entire oocyte cortex, similarly to sqd^{j4B4} mutants, but an additional strong posterior focus is also sometimes visible (E). (G,H) *bcd* RNA localization (purple) to the anterior cortex of stage 8-10 oocytes is not detectably altered in sqd^{j4B4} germline clones (H), in contrast to another oocyte polarity mutant, $mago^{1}/Df(2R)F36$, which shows ectopic posterior *bcd* RNA (G). *sqd* mutant oocytes are smaller than wild-type oocytes at stages 8-10.

Dynein heavy chain (Dhc) is also localized to the posterior pole of wild-type stage 9 oocytes (Fig. 2G) (Li et al., 1994), but it never localized to the posterior pole in sqd^{j4B4} germline clones (Fig. 2H). Thus, in *sqd* mutant oocytes at stages 9-10, localization of posterior factors, including the MT polarity marker kin-β-gal (Fig. 2A), is universally disrupted, suggesting an underlying defect in MT organization.

We therefore examined MT distribution directly with α-tubulin antibody. In wild-type stage 8 and 9 oocytes, α-tubulin staining is higher at the anterior than at the posterior (Fig. 3A), because MTs are nucleated primarily at the anterior cortex (Theurkauf et al., 1992) and are excluded from the posterior pole (Cha et al., 2002). In sqd^{j4B4} germline clones, we detected MTs at uniform density all around the oocyte cortex, indicating that in these mutants the asymmetrical mid-oogenesis MT array does not form correctly (Fig. 3B).

We did not find any combination of *sqd* alleles that shows a significantly greater penetrance of mislocalization for *osk* RNA than for kin-β-gal. In the viable trans-heterozygote, sqd^{j4B4}/sqd^{1}, kin-β-gal was improperly localized at 65% penetrance, either in the center of the oocyte or diffusing away from the posterior pole (n=165; Fig. 2B). This penetrance is comparable to the 65% GFP-Stau mislocalization and 74% *osk* mislocalization observed in oocytes of this genotype (Table 1A and Fig. 2D,F), implying that the mislocalization of *osk* RNA in this and other *sqd* allelic combinations results principally from aberrant MT organization. This is in contrast to, but not mutually exclusive with, the suggestion that Sqd guides *osk* RNA to the posterior by a direct interaction (Norvell et al., 2005). Our observation that GFP-Stau particles cluster aberrantly around the periphery of nurse cell nuclei in *sqd* mutants supports the idea that Sqd affects the behavior of complexes likely to include *osk* RNA, despite the fact that we did not observe any specific effect of Sqd on *osk* RNA localization independent of the MTs.

Cytoplasmic streaming normally occurs at stages 10b-12 and depends on reorganization of the MT cytoskeleton after stage 10a (Theurkauf, 1994; Theurkauf et al., 1992). In *cappuccino* (*capu*) mutants, mislocalization of *osk* RNA is the result of premature cytoplasmic streaming during stages 8-10a (Manseau et al., 1996). In sqd^{j4B4} germline clones, we observed normal streaming at stage 10b but not earlier (data not shown), indicating that the MT arrangements that govern streaming do not depend on Sqd function.

In a subset of polarity mutants, including *PKA*, *bcd* RNA localizes ectopically to the oocyte posterior at stages 8-10; it has been suggested that this reflects localization to an MTOC aberrantly persisting at the oocyte posterior after stage 7 (Lane and Kalderon, 1994). We did not detect ectopic *bcd* at the posterior of sqd^{j4B4} mutant oocytes (n=40, Fig. 3H), even though it was readily observed in $PKA\text{-}C1^{H2}$ germline clones (31%, n=26, data not shown), as well as in another polarity mutant, *mago nashi* (*mago*; Fig. 3G).

Partial MT depolymerization in *sqd* and *PKA* mutants

To further investigate the nature of the MT defects in *sqd* and *PKA* mutants, we examined MTs with α-tubulin antibody following partial depolymerization with colchicine. In wild-type stage 8 and 9 oocytes, MT stubs emanate mostly from the anterior cortex following partial depolymerization (Fig. 3C),

reflecting the presence of an anterior MTOC after stage 7 (Theurkauf et al., 1992). By contrast, partial MT depolymerization in *sqd*^{j4B4} germline clones revealed MTs emanating from the entire oocyte cortex at stages 8 and 9 (Fig. 3D,F). Likewise, in *PKA-C1*^{H2} germline clones, MT stubs could be detected all around the oocyte cortex following partial depolymerization at stages 8-10. Additionally, however, a strong focus of MTs sometimes could be detected at the posterior pole of *PKA-C1*^{H2} germline clones (Fig. 3E), whereas such staining was never seen in *sqd*^{j4B4} germline clones. This suggests that the whole cortex, including the posterior pole, nucleates MTs in both *sqd* and *PKA* mutants but that only *PKA* mutants retain a discrete posterior MTOC. The cortical MTs that persist at the posterior pole in *sqd* mutants presumably do not represent a true organizing center, as they are not capable of supporting ectopic posterior *bcd* RNA, in contrast to those of the *PKA* mutant.

Germline mutations in *sqd* do not affect posterior follicle cell fate specification

Sqd has been shown to affect *grk* RNA localization and translation in mid- to late oogenesis (Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1996; Norvell et al., 1999). If *grk* were also affected in early oogenesis and unable to signal to the posterior follicle cells, this could account for the mid-oogenesis AP polarity defect (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In wild-type oocytes, Grk forms a cap at the posterior of stage 2-6 oocytes (Neuman-Silberberg and Schupbach, 1993) (Fig. 4A). We observed normal Grk localization in only 48% of *sqd*^{j4B4} germline clones at those stages, with Grk localized diffusely throughout the ooplasm in the remaining cases (*n*=86; Fig. 4B).

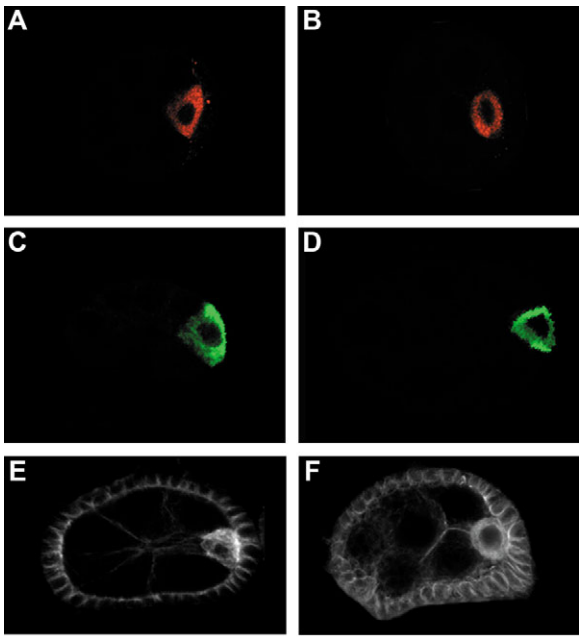

Fig. 4. *sqd* mutant oocytes display polarity defects in early oogenesis. In early wild-type egg chambers, Grk protein (A, red), Orb protein (C, green) and α-tubulin (E) form a cap at the oocyte posterior, while in *sqd*^{j4B4} germline clones, Grk (B, red), Orb (D, green) and α-tubulin (F) are more evenly distributed throughout the cytoplasm of stage 2-6 oocytes.

To test whether early Grk signaling suffices to specify posterior follicle cells in *sqd*^{j4B4} germline clones, we examined two established markers of follicle cell fate. Expression of the posterior follicle cell marker, *pnt*^{rm254}, was normal in egg chambers where the germline was mutant for *sqd* (Fig. 6A). Additionally, we did not see ectopic expression of the anterior centripetal cell marker, *BB127*, in posterior follicle cells surrounding *sqd* mutant oocytes (Fig. 6B). Thus, the early *grk* signal, which is required for *pnt*^{rm254} expression, is transmitted appropriately from *sqd* mutant oocytes, and we conclude that the mid-oogenesis phenotype of *sqd* oocytes is not the result of an earlier defect in *grk* signaling or in posterior follicle cell specification.

Although the anterior and posterior follicle cells are specified correctly in egg chambers containing *sqd*^{j4B4} germline clones, the migration of the anterior border and centripetal cells in those egg chambers was defective at high penetrance (see Fig. S2F in the supplementary material).

sqd mutants display defects in early oocyte polarity

The mislocalization of Grk protein in early *sqd* mutant oocytes led us to question whether early *sqd* oocytes display other polarity defects. Like Grk, Orb protein and *osk* RNA localize in a cap at the posterior of wild-type oocytes in stages 2-6 (Fig. 4C and data not shown). As was found for Grk, Orb was diffusely localized in the ooplasm of early *sqd* mutants, at variable penetrance ranging from 12-52% (*n*=339; Fig. 4D); the penetrance increased with the age of the females, as was the case for various other defects (see below). *osk* RNA was also mislocalized in a manner similar to Orb and Grk in a subset of early oocytes (data not shown). Likewise, stage 2-6 *sqd*^{j4B4} oocytes often had an even distribution of MTs throughout their cytoplasm (Fig. 4F), in contrast to wild-type oocytes, which have a strong focus of tubulin staining at the posterior, indicative of the posterior MTOC (Fig. 4E). Thus, *sqd* mutants display abnormal distributions of germline MTs in both early and mid-oogenesis.

sqd mutants also display defects in oocyte specification and cystocyte mitosis

Ovarioles containing *sqd*^{j4B4} germline clones exhibited a number of additional phenotypes likely to originate in the germarium. These increased in penetrance over time, presumably reflecting progressive depletion of perduring Sqd gene products below functional thresholds in the germarium. Thus, for animals with germline clones induced during the third larval instar, the penetrance of these phenotypes collectively increased from 17% of early egg chambers in 1-3-day-old females (*n*=127; 6-8 days after clone induction) to 89% in 10-12-day-old females (*n*=280; 15-17 days after clone induction).

The most prominent of these phenotypes was the absence of stalks separating adjacent egg chambers (see Fig. S2B in the supplementary material). Also, many egg chambers contained aberrant numbers of germ cells, from as few as two (see Fig. S2C in the supplementary material) to well in excess of 16. Furthermore, the number of germ cells in adjacent egg chambers generally did not add up to 16, nor were excess germ cells present in multiples of 16. Cysts with 16 nurse cells and

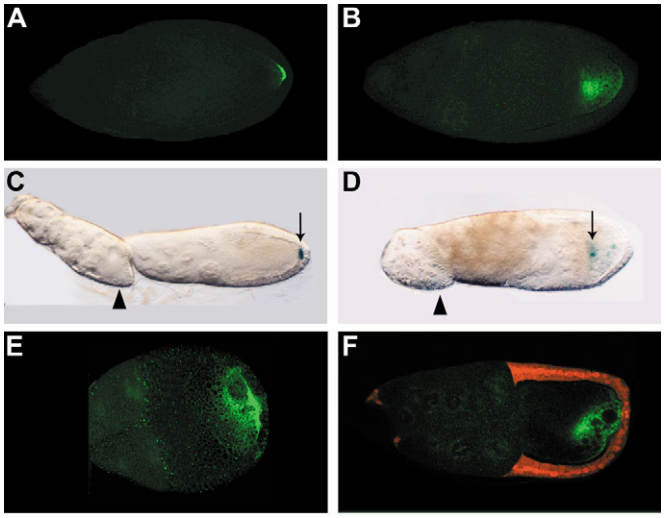

Fig. 5. Both *sqd* and *PKA-C1* mutants display ectopic Osk protein localization. (A,B) Osk protein (green) is detectable only at the posterior of wild-type stage 9 oocytes (A). By contrast, Osk protein is seen in some sqd^{j4B4} germline clone oocytes mislocalized away from the posterior (B). (C,D) Expression of a *lacZ* reporter for *osk* translation, $m1^{414}lacwt$ (blue), is limited to the posterior pole of stage 9 and older wild-type oocytes (C, arrow); it is not expressed in younger wild-type oocytes (arrowhead). In sqd^{j4B4} germline clones, expression of $m1^{414}lacwt$ is detected in the middle of stage 9 oocytes (D, arrow), although it is never expressed earlier (arrowhead). (E,F) Ectopic Osk protein (green) is seen in the middle of some oocytes that are homozygous for a *PKA-C1* null allele (E) or for both sqd^{j4B4} and a *PKA-C1* null (F). Lack of GFP (red) in the germline marks the *PKA-C1* homozygote in (F).

no oocyte were also observed (data not shown), although more commonly, cysts with no oocyte also contained abnormal numbers of germ cells (see Fig. S2B in the supplementary material). Additionally, in egg chambers containing the proper complement of nurse cells and an oocyte, the oocyte was sometimes mispositioned within the cyst, failing to reside at the posterior (see Fig. S2D in the supplementary material). In 10-12-day-old females, a number of egg chambers beyond stage 6 were seen to degenerate. The scoring of defects in AP polarity was always performed using young females and only included egg chambers with 16 germ cells and a normally positioned oocyte.

Translation of mislocalized *osk* RNA in *sqd* and *PKA* mutants

Normally, *osk* RNA is translationally repressed until it becomes localized to its final destination at the posterior pole. Consequently, Osk protein can be detected only at the posterior of stage 9-10 oocytes (Kim-Ha et al., 1995) (Fig. 5A). In sqd^{j4B4} germline clones, Osk protein was seen in the center of stage 9 and 10 oocytes at 29% penetrance (Fig. 5B); no Osk protein was visible in 59% of mutant oocytes, and in the remaining cases (12%) Osk protein was detected only at the posterior (n=259). We never observed ectopic *osk* translation in *sqd* oocytes before stage 9 or in the nurse cells at any stage. An *osk* translational reporter, $m1^{414}lacwt$, which carries the *osk* 5′ and 3′ UTRs flanking the *lacZ* gene (Gunkel et al., 1998), confirms the observations made with Osk

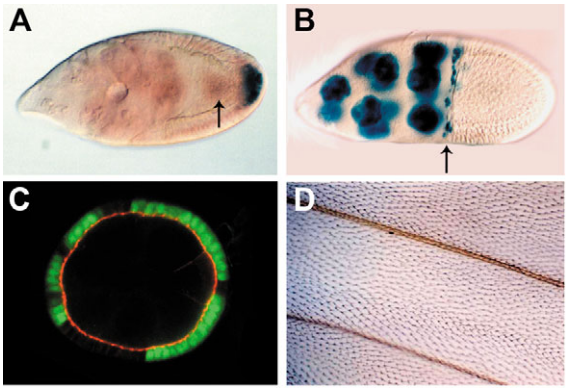

Fig. 6. Loss of Sqd in the germline does not affect posterior follicle cell specification, and loss of Sqd in somatic cells does not affect their polarity. (A) Expression of the posterior follicle cell marker pnt^{rm254} (blue) is normal in sqd^{j4B4} germline clones, while kin-β-gal is mislocalized (arrow). (B) The centripetal follicle cell marker *BB127* (blue) is expressed normally at the anterior of sqd^{j4B4} germline clones (arrow). (C) Baz protein (red) localizes to the apical membrane of follicle cells. The absence of nuclear GFP (green) marks sqd^{j4B4} mutant clones. Note that this egg chamber is also mutant for *sqd* in the germline. Baz localization is normal in sqd^{j4B4} follicle cell clones. (D) The wing blade trichome bristles display normal planar cell polarity in $sqd^{j4B4}/Df(3R)urd$ escaper flies.

antibody. sqd^{j4B4} germline clones showed 34% ectopic β-galactosidase localization in stages 9-10 (n=58) but no ectopic expression in earlier stages (Fig. 5D). Recently, it was reported that excessive PKA activity can result in *osk* translation before the RNA is localized, whereas translation of normally localized *osk* can be reduced by decreasing PKA activity (Yoshida et al., 2004). This led to the suggestion that PKA is required for *osk* translation. We therefore looked at Osk protein in germline clones that were doubly mutant for both $PKA\text{-}C1^{H2}$ and sqd^{j4B4}. Mislocalized Osk was still seen in 27% of *PKA-C1*, *sqd* double mutants (n=15, Fig. 5F), compared with 18% of *sqd* single mutant sibling controls in this experiment (n=130). Furthermore, we also found ectopic Osk away from the posterior in 9% of $PKA\text{-}C1^{H2}$ mutant oocytes at stages 9 and 10, even in the absence of any *sqd* mutation (Fig. 5E). Most $PKA\text{-}C1^{H2}$ mutant oocytes had no detectable Osk, although 24% had Osk solely at the posterior (n=164). Thus, the absence of PKA in the germline does not prevent Osk translation, either at the posterior or in the center of stage 9-10 oocytes.

sqd does not affect polarity in somatic cells

To determine whether Sqd affects polarity of somatic cells in addition to its role in polarizing the oocyte, we examined the localization of several markers of apical polarity in sqd^{j4B4} follicle cell clones. We did not detect any effect on the localization of the apical proteins Notch (N), Bazooka (baz), or Atypical protein kinase C (aPKC) in *sqd* mutant follicle cell clones, even as long as 15 days after clone induction (Fig. 6C). Additionally, we did not detect any defects in planar cell polarity in the trichome bristles of the wing blade, either in sqd^{j4B4} clones or in the wings of sqd^{j4B4}/Df escaper flies (Fig. 6D), and no planar polarity or other defects were apparent in sqd^{j4B4} homozygous eye clones (data not shown). Thus, Sqd

acts specifically in the female germline to organize MTs and establish polarity.

Discussion

Sqd is essential for AP axis formation in the *Drosophila* oocyte

It is well established that the hnRNP Sqd participates in *Drosophila* DV axis formation (Kelley, 1993; Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1996; Norvell et al., 1999). Following a screen to identify factors that interact with PKA in mid-oogenesis, we discovered that Sqd is essential also for AP axis formation. The localization of posterior factors, including *osk* RNA, GFP-Stau, kin-β-gal, and Dhc, was disrupted at stages 9-10 in all *sqd* allelic combinations tested, was highly penetrant in strong *sqd* alleles, and could be rescued by expression of a Sqd cDNA transgene. These defects can be attributed to the failure of *sqd* mutants to establish a normally polarized MT array at mid-oogenesis. We also observed defects in MT organization and in the localization of posterior factors, including Grk protein, in *sqd* mutant oocytes at stages 2-6. Despite the imperfect localization of Grk before stage 6, posterior fate appears to be specified normally in the follicle cells overlying *sqd* mutant oocytes. Thus, *sqd* mutations affect germline-specific processes required for the polarization of MTs in both early and mid-oogenesis.

Loss of PKA in the germline did not affect MT polarity in early oogenesis, as judged by Orb localization in stages 2-6 (data not shown), but, similarly to *sqd* mutants, it disrupted MT polarity in mid-oogenesis without discernibly altering follicle cell fate. However, despite several similarities, we observed a significant difference in the MT organization of *sqd* and *PKA* mutants at stages 8-10, as discussed in greater detail below.

MT organization in polarity mutants

In *grk* and *cni* mutants, where the posterior follicle cells do not differentiate properly, both the subsequent RNA localization defects and the failure of the oocyte nucleus to migrate from the posterior to the anterior have been attributed to defects in MT reorganization resulting from loss of a posterior follicle cell signal (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In *PKA* and *sqd* mutant oocytes, the anterior migration of the oocyte nucleus, which depends on MT function (Koch and Spitzer, 1983), was unaffected, despite the accompanying MT defects and the highly penetrant mislocalization of *osk* RNA and other posterior factors. Thus, it appears that either discrete aspects of the MT organization, which direct nucleus migration, are spared in *PKA* and *sqd* mutants or the overall disruption of MT organization by loss of PKA or Sqd is simply less severe than that caused by loss of posterior follicle cell fate.

The normal organization of MTs in stage 8-10 oocytes is not entirely clear. In addition to MTs nucleated at the anterior cortex, MTs have been proposed to emanate from all cortical positions, with the exception of the posterior pole (Cha et al., 2001; Cha et al., 2002). This assertion is based on the observations that components associated with MT minus-ends, such as γ-tubulin and the centrosome component Centrosomin (Cnn) (Cha et al., 2002), can be seen along the entire oocyte cortex, and that injected *bcd* RNA localizes to the lateral cortices as well as the anterior, but not to the posterior pole (Cha et al., 2001). Hence, normal posterior localization of *osk* RNA may require the clearing of MTs nucleated both from a discrete posterior MTOC established before stage 6 and from dispersed cortical sites established after stage 7.

Staining with α-tubulin antibody following partial MT depolymerization revealed MT stubs emanating mostly from the anterior in wild-type oocytes, whereas *PKA* and *sqd* mutant oocytes retained short MTs around the entire oocyte cortex, including the posterior pole. Some *PKA* mutant oocytes also showed an elevated posterior concentration of MTs not seen in *sqd* mutant oocytes. Thus, it appears that the primary MT defect in *sqd* mutants is the failure to eliminate cortical sites of MT nucleation beyond stage 7, whereas *PKA* mutants additionally retain a posterior MTOC beyond stage 6. This hypothesis can explain why ectopic *bcd* RNA localizes at the posterior of *PKA* mutant oocytes but not *sqd* mutant oocytes. It should, however, be noted that since classical MTOC components, such as γ-tubulin, are present along the entire oocyte cortex at stages 9-10 even in wild-type oocytes (Cha et al., 2002) (J, Steinhauer, unpublished data), the inference of a discrete posterior MTOC from partial MT depolymerization experiments cannot be confirmed directly.

sqd mutants display polarity defects in early oogenesis

In a proportion of *sqd* mutant stage 2-6 oocytes, Grk, Orb, *osk* RNA and MTs were distributed evenly throughout the ooplasm rather than localizing in a cap at the oocyte posterior. Although these defects did not appear to cause the subsequent AP defects by preventing posterior follicle cell specification, we cannot rule out the possibility that the early and late polarity phenotypes are causally related in some other way. For instance, it is possible that a molecule(s) required at the posterior of the oocyte for the MT reorganization at stages 7-8 is improperly localized by stage 6 in *sqd* mutants, as are Grk, Orb and *osk* RNA. If MT rearrangements were very sensitive to the localized concentration of this hypothetical regulator, an early polarity defect of apparently low penetrance could be translated into a much more penetrant polarity phenotype at mid-oogenesis.

sqd is not the only mutant to cause polarity defects in both early and mid-oogenesis. For example, defects in early polarity are caused by mutations in Armitage (Armi), a component of the RNA silencing machinery, and these defects were proposed to be the cause of a mid-oogenesis AP polarity phenotype (Cook et al., 2004). However, we found that $pnt^{998/12}$ expression was not disrupted in $armi^{1}$ homozygotes (J. Steinhauer, unpublished). Weak *par-1* alleles also affect mid-oogenesis polarity without affecting posterior follicle cell fate (Shulman et al., 2000), whereas strong alleles disrupt early polarity severely, causing oocyte identity to be lost (Cox et al., 2001; Huynh et al., 2001). Thus, for several mutations, including *sqd*, *armi* and *par-1*, it is unclear whether MT organization is disrupted independently at two distinct phases of development or whether there is a causal connection between the early and later polarity phenotypes that is not evident as a failure in posterior follicle cell specification. In either case, a single molecular target might account for both the early and mid-oogenesis phenotypes.

Additional early *sqd* phenotypes

Several additional phenotypes became prevalent in older sqd^{j4B4} germline clones, rising to very high penetrances after 2 weeks. Among the varied late onset *sqd* phenotypes, the oocyte sometimes was mispositioned within the egg chamber, even in those egg chambers containing the normal complement of nurse cells to oocyte. This phenotype can arise in several ways, including as a result of delayed oocyte specification (Gonzalez-Reyes et al., 1997). A role for Sqd in oocyte specification is supported by the presence of cysts with 16 nurse cells and no oocyte in these older ovarioles. In other cases, cysts with fewer than 16 germ cells were observed, implicating Sqd in cystocyte mitosis. Both oocyte specification and the normal cystocyte divisions depend on specific arrangements of the MT cytoskeleton in the germarium (Huynh and St Johnston, 2004). Thus, it is likely that some of these late onset *sqd* phenotypes, like the polarity phenotypes, are caused by a defect in regulating MT dynamics.

osk translation in polarity mutants

In sqd^{j4B4} germline clones, we noticed the accumulation of Osk protein in the cytoplasm of stage 9-10 oocytes. A similar observation was reported for mutations in another hnRNP A/B family member, Hrp48 (Hrb27C – FlyBase) (Yano et al., 2004). Normally, Osk protein accrues only at the posterior cortex, and translation of *osk* RNA is presumably repressed elsewhere (Kim-Ha et al., 1995). Therefore, loss of *sqd* may cause de-repression of *osk* translation. However, we did not see ectopic *osk* translation in stage 6-8 *sqd* mutant oocytes, detected either with Osk antibody or with an *osk* translation reporter, in contrast to the premature expression observed with the *osk* translation reporter in *hrp48* mutants (Yano et al., 2004) or with similar reporters lacking specific repressor elements (Gunkel et al., 1998; Kim-Ha et al., 1995). Thus, although we do not discount the idea that *sqd* is directly involved in translational regulation of *osk*, we propose an alternative hypothesis for the ectopic Osk protein accumulation in *sqd* mutants. As most of the posterior components that we examined were mislocalized to the center of sqd^{j4B4} oocytes, we believe that the primary AP defect in *sqd* mutants is that the MT plus-ends are focused incorrectly at the center of the oocyte. Hence, all the necessary components for *osk* translation may be localized together, and we hypothesize that the *osk* translation machinery is assembled and activated in the middle of the *sqd* mutants as it normally is at the posterior of wild-type oocytes at stage 9.

We also detected a low penetrance of ectopic Osk protein in *PKA* mutants. The scenario outlined above could be true for *PKA* mutants as well. Regardless of the mechanism, it is clear from this result that PKA is not absolutely required for Osk translation, although it may enhance *osk* translation, as previously suggested (Yoshida et al., 2004).

Targets of PKA and Sqd

Although *sqd* was identified in a screen for modifiers of *PKA* in oocyte polarity, retesting with various alleles indicated that there is not a strong genetic interaction between the two loci (data not shown). Both Sqd and PKA act in mid-oogenesis to reorganize the oocyte MTs in response to a normal posterior follicle cell signal, but specific MT defects differ between the two mutants, as discussed above. Thus, they probably have different targets and mechanisms in this complex process.

The hnRNP Sqd is an RNA-binding protein. Another hnRNP of the same family, Hrp48, is also required for MT reorganization at mid-oogenesis (Yano et al., 2004). Sqd and Hrp48 bind each other in vitro, cooperate in *grk* RNA localization (Goodrich et al., 2004) and have similar localization patterns throughout oogenesis (Matunis et al., 1994; Yano et al., 2004) (see Fig. S1G in the supplementary material). Thus, one might expect these two proteins to act together in MT reorganization. Although we were unable to detect a strong genetic interaction between *sqd* and *hrp48* in AP polarity (data not shown), we speculate that they are collectively necessary for the localization and translation of one or a small number of specific RNA molecules required for MT repolarization at mid-oogenesis.

hnRNPs normally participate in the processing of many RNAs (Dreyfuss et al., 2002), but their generic functions may be partially redundant, so that, for example, in *sqd* mutants, continued cell viability is not impaired despite the presence of a strong AP polarity defect. Our ability to induce large, persistent somatic cell clones for sqd^{j4B4} without causing any polarity or other phenotypes supports this idea. Follicle cell polarity was also normal in *PKA* mutant clones (data not shown). Thus, the disruptions in MT polarity that we observe for both *PKA* and *sqd* mutants represent specialized functions of these proteins in germline cells.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/24/5515/DC1

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Table S1. A constitutively active PKA transgene (*actp>mC) fails to rescue embryonic and larval lethality of *PKA* mutants efficiently**

A

Germline genotype	Eggs (*n*)	Eggs/ female/day	% Hatching		% Hatched larvae surviving to at least						Paternal rescue
					Second instar		Pupae		Adults		
			–	+	–	+	–	+	–	+	
+/+	934		63	63	>90	>90	>90	>90	>90	>90	
	928	2.7									
DC0^{H2}/DC0^{H2}	438		0	0							
	566	0.8	0	0							
*DC0^{H2}/DC0^{H2}, actp>mC**	2838		1.1	1.6	39	83	0	55	0	55	
	3033	0.8	1.7	3.3							
	3590		0.6	1.2							

B

Zygotic genotype	*n*	% Hatched larvae surviving to at least			
		Second instar	Third instar	Pupae	Adults
DC0^{H2}/Df(2L)γ15	200	0	0	0	0
*DC0^{H2}/Df(2L)γ15, actp>mC**	279	12	0	0	0
*DC0^{H2}/+, actp>mC**	266	43	21	11	3
actp>mC/+*	130	49	30	17	10