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An A-kinase anchoring protein is required for Protein kinase A regulatory subunit localization and morphology of actin structures during oogenesis in *Drosophila*

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SUMMARY

Protein kinase A (PKA) holoenzyme is anchored to specific subcellular regions by interactions between regulatory subunits (Pka-R) and A-kinase anchoring proteins (AKAPs). We examine the functional importance of PKA anchoring during *Drosophila* oogenesis by analyzing membrane integrity and actin structures in mutants with disruptions in Akap200, an AKAP. In wild-type ovaries, Pka-RII and Akap200 localized to membranes and to the outer rim of ring canals, actin-rich structures that connect germline cells. In *Akap200* mutant ovaries, Pka-RII membrane localization decreased, leading to a destabilization of membrane structures and the formation of binucleate nurse cells. Defects in membrane integrity could be mimicked by expressing a constitutively active PKA catalytic subunit (Pka-C) throughout germline cells. Unexpectedly, nurse cells in *Akap200* mutant ovaries also had enlarged, thin ring canals. In contrast, overexpressing Akap200 in the germline resulted in thicker, smaller ring canals. To investigate the role of *Akap200* in regulating ring canal growth, we examined genetic interactions with other genes that are known to regulate ring canal morphology. *Akap200* mutations suppressed the small ring canal phenotype produced by *Src64B* mutants, linking *Akap200* with the non-receptor tyrosine kinase pathway. Together, these results provide the first evidence that PKA localization is required for morphogenesis of actin structures in an intact organism.

Key words: Actin, Protein kinase A, AKAP, Oogenesis, Src64B

INTRODUCTION

During development, cell function and morphology are influenced by exposure to myriad extracellular signals. Protein kinase A, a ubiquitous, highly conserved serine-threonine kinase, is a key intracellular transducer of many hormonal and other extracellular signals. In the absence of cAMP, inactive Protein kinase A is a holoenzyme (PKA), a heterotetramer of two identical catalytic subunits (Pka-C) and two identical regulatory subunits (Pka-R). When cAMP is present, it binds to the regulatory subunits and releases catalytic subunits from the holoenzyme, allowing phosphorylation of target substrates. In metazoans (except *C. elegans*) (Gross et al., 1990; Lu et al., 1990), the PKA regulatory subunits are of two types: type I (Pka-RI) or type II (Pka-RII). Mammals have two isoforms of each type of regulatory subunit (e.g. Pka-RIα or Pka-RIβ) but *Drosophila* has only one isoform of each type of regulatory subunit (Kalderon and Rubin, 1988). Once activated, the catalytic subunits are capable of phosphorylating a large number of protein substrates, both in vitro and in vivo.

The promiscuous enzymatic activity of the Pka-C subunit raises questions as to how cAMP-mediated signaling can achieve specific cellular responses. One proposed mechanism is that PKA holoenzyme is regulated by sequestration near specific targets, ensuring a rapid local response to an extracellular signal (Mochly-Rosen, 1995). This sequestration is achieved through A-kinase anchoring proteins (AKAPs), a heterogeneous family of proteins that bind to PKA regulatory subunits and anchor PKA holoenzyme (Scott and McCartney, 1994). Many different subcellular regions are targets for PKA anchoring (Colledge and Scott, 1999). AKAPs were thought to interact only with the type II regulatory subunit; recent evidence suggests, however, that Pka-RI can interact with dual-specificity AKAPs that bind to either Pka-RI or Pka-RII (Huang et al., 1997). In mammalian cells, the function of AKAPs can be disrupted by treatment with Ht31, a dominant negative peptide that interferes with AKAP–Pka-RII interactions (Carr et al., 1991). Disrupting PKA localization with Ht31 interfered with several cellular functions, including the PKA-dependent regulation of L-type calcium channels in HEK293 cells (Gao et al., 1997) and skeletal muscle cells (Johnson et al., 1994), AMPA or kainate receptor potentiation in hippocampal neurons (Rosenmund et al., 1994), calcium-activated potassium channels in isolated smooth muscle patches (Wang and Kotlikoff, 1996), aquaporin channel

function in pancreatic cells (Klussmann et al., 1999) and apoptotic pathways (Harada et al., 1999). These analyses reveal the importance of AKAP–Pka-RII interactions and support the hypothesis that sequestration of PKA may facilitate localized and/or specific response to cAMP signaling. Here, we test the possibility that such localization is necessary for regulating actin cytoskeletal dynamics and for maintaining tissue integrity during development.

In *Drosophila*, two AKAPs were cloned using in vitro gel overlay assays. *Akap550* encodes a protein that has no known functional domains other than a coiled-coil Pka-RII binding site (Han et al., 1997). Immunocytochemical analysis of Akap550 showed that it is present in the cytoplasm of developing gut, trachea and salivary gland cells and is particularly elevated in the nervous system (Han et al., 1997) and in other adult tissues (S. M. J. and C. A. B., unpublished observations). The second AKAP, Akap200, contains both a coiled-coil Pka-RII binding domain and a myristoylated alanine-rich C-kinase substrate (MARCKS) domain (Li et al., 1999). Alternate splicing of this gene produces two transcripts; one contains the Pka-RII binding domain and one lacks it, but the MARCKS domain is retained in both. Akap200 protein associates with membranes and subcortical regions of cells when transfected into cultured Schneider cells, and purified Akap200 binds to purified actin filaments in vitro (Rossi et al., 1999). These observations suggest that Akap200 is involved in linking PKA signaling to the actin cytoskeleton.

Although Akap200 is expressed throughout development in the fly (Li et al., 1999) (S. M. J. and C. A. B., unpublished observations), its function is not known. *Drosophila* oogenesis provides an ideal model system for analyzing AKAP-mediated regulation of PKA signaling events in a developmental context. The egg chambers present in the *Drosophila* ovary contain large cells with well-defined and easily observed actin-derived structures. Furthermore, large numbers of egg chambers are easy to obtain and egg chambers at different stages of development are present within a single ovary. Oogenesis begins in the germarium, a structure at the anterior tip of each ovariole that contains the germline and somatic precursor cells (Spradling, 1993). Approximately halfway down the germarium, interleaving follicle cells migrate to surround a single germline cyst and form an egg chamber. Thus, each egg chamber consists of an epithelial monolayer of somatically-derived follicle cells surrounding a syncytial cyst of 16 germline-derived cells. The germline cyst arises by four incomplete cytokineses of a germline cystoblast cell and contains 15 polyploid nurse cells and a single diploid oocyte. The nurse cells and oocyte are connected to each other via specialized channels known as ring canals. These ring canals are rich in filamentous actin as well as several other proteins that regulate the morphology and growth of the ring canals during oogenesis (Cooley, 1998).

In this study, we describe our investigation of *Akap200* function during oogenesis. We show that this gene contributes to the maintenance and regulation of cytoskeletal structures in the germline cells. Our results demonstrate that PKA anchoring is necessary to maintain the cytoarchitecture of the germline-derived cells, providing evidence that PKA anchoring is important during morphogenesis.

MATERIALS AND METHODS

Fly stocks

All flies were raised on standard cornmeal-molasses-agar food at 25°C. In this study, we used w^{1118} flies as wild type. The stocks *l(2)k07118*, *EP2254*, *UAS-GFPnls*, *nos-GAL4-VP16* and *NGT40* are described in FlyBase (FlyBase, 1999).

Akap200^{k07118} excision alleles were generated in two ways:

(1) To generate imprecise excisions, we crossed *y w*; *Akap200*^{k07118}*/CyO* females to *w; Sp/CyO; Δ2-3 Sb/ TM6* males. *y w; Akap200*^{k07118}*/CyO; Δ2-3 Sb/+* male progeny were then mated to w^{1118} females. From these progeny, individual *w*; *Akap200*$^{\Delta k07118}$*/+; +/+* males were mated to *Df(2L)N22-14/CyO* females, and progeny were scored for lethality and extra notal bristles. *ΔAkap200*^{k07118}*/Df(2L)N22-14* males from lines that had extra bristles, partial lethality or other abnormalities, were crossed to *y w; Pin/CyO* females. Individual long-bristled, Curly males (containing either the *Df(2L)N22-14* or the *ΔAkap200*^{k07118} chromosome) were retested by crossing back to *Df(2L)N22-14/CyO* females, and scoring as above. These individual males were stored at 18°C until reproducibility was established; stocks were generated from males that produced a consistent phenotype in the backcross to *Df(2L)N22-14*. We hypothesized that null alleles would result in complete penetrance of the lethality or bristle-duplication phenotypes. Although we screened a total of 88 *w* excision lines, none showed complete penetrance of either phenotype. Importantly, loss of all detectable immunoreactivity in situ or on western blots (data not shown) failed to produce strains that were completely penetrant for the viability or bristle phenotypes. Thus, the null phenotype may not be completely penetrant for these phenotypes, and molecular screening will be necessary to isolate deletion mutations that remove the coding region. Given the position of the *P* element in the locus, such deletions are expected to be rare events. We did select four new alleles for further analysis (see Table 1): *Akap200*^{ix4}, viable, infrequent bristle duplications; *Akap200*^{ix1}, slight decrease in viability, frequent bristle duplications; *Akap200*^{ix2}, decreased viability, infrequent bristle duplications; *Akap200*$^{\Delta 7}$, decreased viability, frequent bristle duplications. The analyses of all *Akap200* mutants were performed using *Akap200*/*Df(2L)N22-14* females; the *Df(2L)N22-14* (29C1-2;30C8-9;30D1-2;31A1-2) deficiency removes the Akap200 locus completely.

(2) To generate precise excisions, we followed the scheme described above, except that *y w; Akap200*^{k07118}*/CyO; Δ2-3 Sb/+* males were mated to *y w; Pin/CyO* females. From these progeny, individual *y w;* Δ*Akap200/Pin* were mated to *Df(2L)N22-14/CyO* females, and Δ*Akap200*/*Df(2L)N22-14* flies were scored as above. Stocks were established from lines that lacked extra bristles. This screen also produced imprecise excisions that resulted in viability and bristle defects, but again, none were completely penetrant.

Molecular biology

The structure of the *Akap200* locus was derived from cDNAs reported in (Li et al., 1999). The extent of the *Akap200* deletions in the imprecise excision alleles was determined by preparing genomic DNA from w^{1118} or *Akap200*/*Df(2L)N22-14* flies and analyzing the DNA by Southern analysis according to established techniques (Jowett, 1998; Sambrook et al., 1989) and manufacturer's instructions (Genius Kit, Roche Diagnostics, Mannheim, Germany).

Immunocytochemistry

Adult females were placed in vials with fresh yeast and males for several days before dissecting. Ovaries were dissected, processed and analyzed as described previously (Jackson and Berg, 1999). Antibody concentrations were as follows: anti-phosphotyrosine 1:10 (Zymed, South San Francisco, CA, USA), anti-*Drosophila* Hts-RC 1:10 (gift from L. Cooley), anti-*Drosophila* Pka-RII 1:2500 (gift from D. Kalderon), anti-*Drosophila* Akap200 1:1000 (gift from C. Rubin),

Alexa Fluor 488 anti-mouse and Alexa Fluor 568 anti-rabbit 1:100 (Molecular Probes, Eugene, OR, USA). Where appropriate, DAPI (0.2 μg ml^{-1}) and/or fluorescent phalloidin conjugates (2 U ml^{-1} final concentration, Molecular Probes) were included in secondary antibody incubations. Egg chambers were photographed on a Nikon Microphot FXA microscope and slides scanned using an Agfa Arcus II flatbed scanner, or digital images acquired on a BioRad MRC600 laser-scanning confocal microscope. Images were transferred to Adobe Photoshop and adjusted for brightness and contrast.

To measure relative sizes of ring canals, Z-series optical sections through identically staged normal (*w^{1118}* or *w^{1118}/+; Df(2L)N22-14/+* or *daughterlessGAL4/UAS-Akap200*) and mutant egg chambers were acquired by confocal microscopy and projected onto a single flat plane using NIH Image 1.62. To measure the outside diameter, linear measurements of the number of pixels through the widest projection of ring canals were taken using NIH image software. The inside diameter was measured in a similar way through the widest inner diameter that lacked discernable staining; if no inner diameter could be discerned, no measurement of that ring canal was taken. Inner and outer diameters were measured for all ring canals possible in an egg chamber. The number of pixels for all ring canals in an egg chamber were averaged, and then outer diameters in mutants were normalized to outer diameters in control flies, and inner diameters in mutants were normalized to inner diameters in controls (wild-type = 1.00).

RESULTS

Akap200 and Pka-RII are localized to membranes and ring canals

Recent analyses of abnormal nurse cell fusions during *Drosophila* oogenesis revealed that genes regulating cytoskeleton function and genes involved in cAMP metabolism were required to preserve the structural integrity of the nurse cells (Jackson and Berg, 1999; Jackson and Blochlinger, 1997). We hypothesized that the anchoring of PKA holoenzyme near the cortex of cells may regulate cytoskeletal function. To test this hypothesis, we examined the distribution of AKAPs in wild-type ovaries. One of these, Akap200, could be detected near the membranes of the germline cells throughout oogenesis and was therefore a candidate for regulating PKA activity near the subcortical cytoskeleton (Figs 1 and 3). In the somatic follicle cells, Akap200 was also associated with membranes during the early stages of oogenesis. Expression decreased in the oocyte-associated follicle cells as they began their migration over the oocyte at approximately stage nine of oogenesis. Akap200 expression remained in the follicle cells associated with the nurse cells and in the nurse cells themselves (Fig. 1B). We also noticed that Akap200 was enriched on the ring canals of the nurse cells. Ring canals have an inner and outer rim, and although some proteins associate only with inner rims, specific functions have not yet been attributed to either the inner or the outer rim. Nevertheless, costaining with Hts-RC antibodies, which recognize inner-rim-specific epitopes of the *hu-li tai shao* gene product (Robinson et al., 1994), suggested that Akap200 localized to the outer rim of the ring canals (Fig. 1C). Finally, in addition to the membrane and ring canal association, Akap200 immunoreactivity was also associated with cytoplasmic puncta at all observable stages.

The catalytic subunit of Pka-C is localized to the membranes of germline cells in wild-type ovaries (Lane and Kalderon, 1995). The subcellular localization of the regulatory subunits has not yet been described however, and because the catalytic subunits dissociate from the regulatory subunits in the presence of cAMP, the subcellular localization of the regulatory and catalytic subunits may not necessarily overlap. We therefore stained wild-type ovaries with antibodies prepared against the *Drosophila* Pka-RII subunit (Crittenden et al., 1998). In the ovary, the localization of Pka-RII resembled Akap200 (Fig. 1D and E). A majority of Pka-RII was associated with membranes of both the follicle cells and the germline-derived cells throughout oogenesis. At the anteriormost region of the germarium (region 1), Pka-RII was found on the membranes of the germline stem cells (Fig. 1D). Pka-RII levels appeared to be higher in the follicle cells than in the germline cells. In the middle stages of oogenesis, Pka-RII expression increased

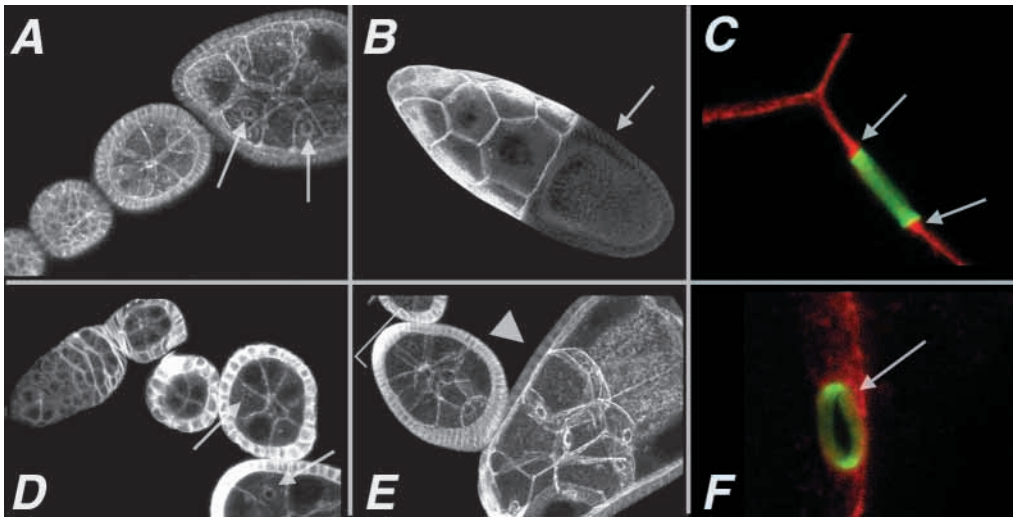

Fig. 1. Akap200 and Pka-RII localization in *Drosophila* wild-type ovaries. (A-C) Akap200 localization. (A) Akap200 protein was present on membranes of follicle cells and nurse cells at all stages of oogenesis. It was also a component of ring canals (arrows). (B) Akap200 expression diminished after stage ten in the follicle cells over the oocyte (arrow), but persisted in the nurse cell-associated follicle cells, nurse cells and ring canals. (C) Akap200 immunoreactivity (red) did not colocalize with Hu-li tai shao immunoreactivity (green). Akap200 was enriched on the outer rim of the ring canal (arrows). (D-F) Pka-RII localization. (D) Germarium and early-staged egg chambers. Like Akap200, Pka-RII protein localized to membranes and ring canals (arrows). (E) Later-staged egg chambers, including a portion of a stage nine egg chamber (arrowhead). Pka-RII was also enriched in a group of follicle cells at the anterior of egg chambers (bracket). (F) Pka-RII immunoreactivity (red) did not colocalize with Hu-li tai shao immunoreactivity (green), suggesting that it was enriched on the outer rim of ring canals.

Table 1. Frequency of extra bristles and binucleate cells in *Akap200* excision lines

Genotype	Molecular lesion	Viability*	*n**	Extra bristles†	*n*†	Normal cells‡	Binucleate cells	Other§	*n*¶
w^{1118}/+; *Df(2L)N22-14*/+		100%	443	2%	188	95%	1.4%	3.6%	440
Akap200^{k07118}/*Df(2L)N22-14*	*PlacW* inserted into first exon	81%	1087*	19%	134	85%	11%	3.2%	784
Akap200^{ix4}/*Df(2L)N22-14*	4 kb internal deletion in *P* element	92%	636	10%	168	82%	16%	1.8%	538
Akap200^{ix1}/*Df(2L)N22-14*	1 kb internal deletion in *P* element	81%	557*	25%	123	79%	18%	2.7%	696
Akap200^{ix2}/*Df(2L)N22-14*	2 kb internal deletion in *P* element	75%	509*	14%	94	73%	24%	3.4%	690
Akap200$^{\Delta 7}$/*Df(2L)N22-14*	*P* element plus 1.2 kb of first *Akap200* intron removed	77%	799*	30%	174	61%	33%	6.1%	885

*Percentage of *Akap200/Df(2L)N22-14* adults compared to Mendelian-expected numbers, * deviation from predicted frequency is significant ($P<<0.001$ by χ^2 test). *n*=total number of adults scored.

†Percentage of *Akap200/Df(2L)N22-14* adults with at least one extra notal bristle, *n*=number of *Akap200* mutant adult cuticles examined.

‡Oogenesis phenotypes were scored between stages two and eleven. Percentages are percent of all egg chambers at these stages with the indicated phenotype.

§Examples of other phenotypes include degenerating egg chambers, cyst encapsulation defects and arrest of egg chamber maturation.

¶*n*=number of egg chambers scored between stages two and eleven.

in the anteriormost group of follicle cells that will become border cells (Fig. 1E, bracket). Pka-RII was also found on the outer rim of ring canals of nurse cells (Fig. 1F). It persisted on the ring canals until oogenesis was completed. Pka-RI localization was diffuse throughout the cytoplasm in all the cells in the ovary, and did not appear to be localized to particular structures in wild-type egg chambers (data not shown).

Identification of a *P* element inserted into the *Akap200* locus

To evaluate the significance of Akap200 localization and to test its function in PKA anchoring, we characterized a *P* element insertion in the *Akap200* locus (*l(2)k07118*^{k07118}), identified and mapped by the Berkeley Drosophila Genome Project (Spradling et al., 1999). This *P* element is located in the large first intron of the *Akap200* gene (Fig. 2), and although the mutation was originally described as lethal, we found that an unrelated background mutation on the chromosome accounted for a majority of the lethality. Once this background lethal mutation was removed, we were able to recover *Akap200* homozygotes at slightly less than Mendelian-predicted frequencies (Table 1); we therefore refer to this allele as *Akap200*^{k07118}. Nevertheless, in all our experiments, we analyzed the phenotypes of hemizygous *Akap200* mutants (*Akap200/Df(2L)N22-14*) to reduce the contribution of other recessive mutations on the original *P* element chromosome. To verify that the *P* element was responsible for Akap200 phenotypes, and to generate other alleles, we mobilized the *P* element and established multiple, independent excision lines. Precise excision reverted all the phenotypes described below, demonstrating that the *P* element was responsible for the observed abnormalities. Several lines exhibited decreased viability and/or the presence of extra notal bristles when trans to the *Df(2L)N22-14* chromosome; we chose four of these lines for further analysis. Although all of these lines produced extra notal bristles, neither the original insertion allele nor any of the excision lines demonstrated a complete penetrance in the reduced viability or bristle phenotype, described below (Table 1 and Fig. 2).

Cuticles and other adult structures from *Akap200*^{k07118}/*Df(2L)N22-14* hemizygotes had a mostly wild-type morphology. In approximately a fifth of the adult flies, however, extra notal macrochaetae were present (Table 1). These extra macrochaetae were accompanied by a socket cell and were either directly adjacent to the normal macrochaete or midway between the proximal and distal macrochaetae. Extra bristles were not observed in other locations, however, and the presence of more than one extra bristle on a single notum was rare. These observations suggest that Akap200 could be

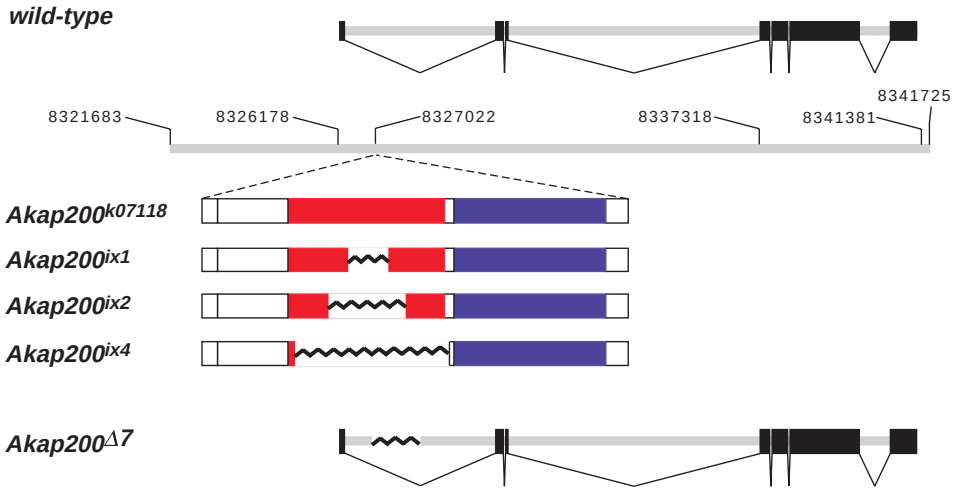

Fig. 2. Molecular characterization of *Akap200* alleles. Exons are depicted as black bars at the top. The BDGP nucleotide number of particular landmarks within contig AE003621 are shown on the bar below, including the *P*-element insertion site into *Akap200* (8327022), start codon (8337328) and polyadenylation site (8341381). The site of the *PlacW* insertion in the first intron is indicated at the confluence of the dashed lines. The *P* element alleles are denoted with blue bars indicating *lacZ* sequences, red bars as *miniwhite* sequences. Sequences removed in the excision lines are indicated with jagged lines.

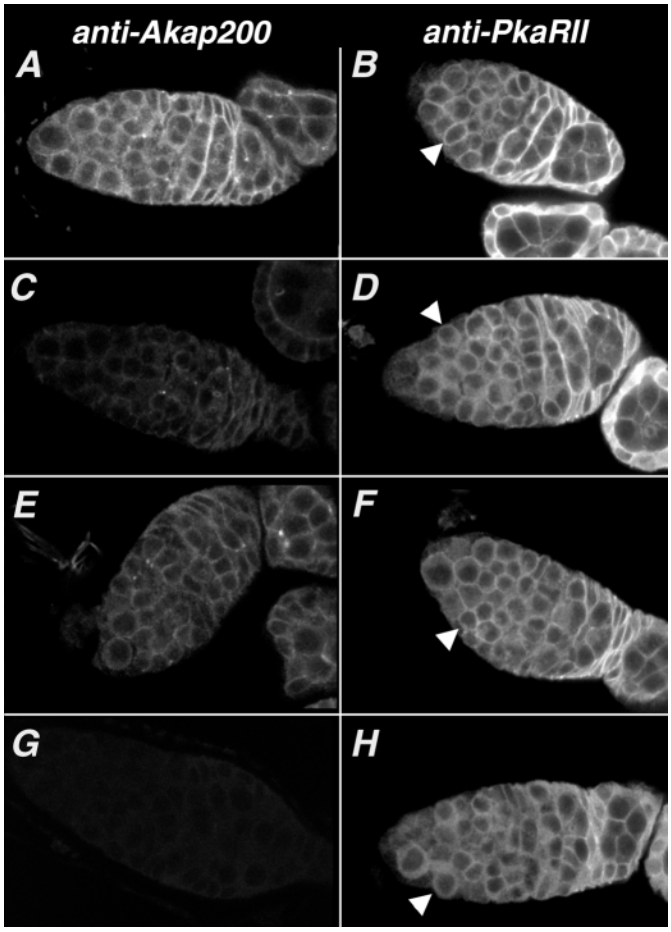

Fig. 3. Pka-RII localization in *Akap200* mutants. (A,B) Control germaria. Akap200 (A) and Pka-RII (B) localized to membranes of germline and follicle cells in the germarium (arrowhead in B). (C-H) Akap200 mutant ovaries. In *Akap200^{k07118}/Df(2L)N22-14* germaria, most of the Akap200 immunoreactivity was lost (C), resulting in a decrease of Pka-RII membrane localization in germline cells (D, arrowhead). Females hemizygous for the excision allele *Akap200^{ix4}/Df(2L)N22-14* exhibited a partial restoration of Akap200 immunoreactivity in the germarium (E). As a result, Pka-RII membrane association was partially restored (F, arrowhead). Females hemizygous for the strongest allele (*Akap200$^{\Delta 7}$/Df(2L)N22-14*) had almost undetectable Akap200 immunoreactivity (G) and decreased Pka-RII membrane localization (H, arrowhead). Pka-RII membrane localization was not completely eliminated by this allele, however. Note that the total amount of Pka-RII protein appeared to be unchanged in all *Akap200* mutants; only the fraction associated with membranes and the fraction found in the cytoplasm changed.

involved in the choice between the alternate cell fates of epidermis or sensory organ.

Akap200 mutants decrease Pka-RII membrane localization

Based on the similar staining patterns, we hypothesized that *Akap200* was responsible for the Pka-RII membrane sequestration and therefore, disrupting *Akap200* function would redistribute Pka-RII to other areas. To test this hypothesis, we examined Pka-RII localization in *Akap200* mutants. In control germaria, Akap200 and Pka-RII are associated with membranes of both the germline and follicle cells (Fig. 3A and B). In *Akap200^{k07118}* mutant ovaries, almost all the Akap200 immunoreactivity was abolished (Fig. 3C). This low but detectable residual staining suggested that the *Akap200^{k07118}* allele was not null. The effects of loss of *Akap200* function were most evident in the germline cells, in which Pka-RII membrane staining decreased and cytoplasmic staining increased (Fig. 3D). Membrane association persisted in the follicle cells however, and to a lesser degree in later-staged nurse cells. This persistent membrane staining may result from partial *Akap200* activity or because other AKAPs anchor Pka-RII to the membranes of these cells. In the excision allele *Akap200^{ix4}*, a partial restoration of Akap200 immunoreactivity (Fig. 3E) correlated with a partial restoration of Pka-RII membrane localization, with a concomitant decrease in cytoplasmic staining (Fig. 3F). In *Akap200$^{\Delta 7}$* flies, Akap200 immunoreactivity is undetectable (Fig. 3G) and is indistinguishable from controls with secondary antibody only (data not shown). As a result, Pka-RII membrane association is reduced in the germline stem cells and in other cells of the ovary (Fig. 3H). Notably, the total amount of Pka-RII protein was unchanged in each of the *Akap200* mutants. Akap200 is therefore responsible for sequestering a majority of Pka-RII to the membranes of germline cells during the early stages of oogenesis.

Akap200 mutants produce binucleate nurse cells

Our hypothesis predicts that if PKA anchoring is important for preserving nurse cell membrane and actin integrity, then disrupting anchoring should produce binucleate nurse cells. We used both loss-of-function and gain-of-function approaches to test this prediction. First, we analyzed the effects of *Akap200* mutations on the morphology of the egg chamber by staining mutant egg chambers with fluorescent-phalloidin conjugates, to examine actin-rich ring canals and subcortical actin, and DAPI, to visualize the nuclei. Females mutant for *Akap200* produced egg chambers that had multinucleate cells with ring canal remnants (Table 1, Fig. 4A and B). As is the case with most other mutations that produce multinucleate cells, these remnants stain with Hts-RC antibodies (data not shown), suggesting that cytokineses and ring canal formation were initially normal in these mutants.

Second, we reasoned that loss of *Akap200* activity and the resulting failure to sequester PKA holoenzyme would produce a change in the local concentration of available Pka-C. We tested this hypothesis directly by expressing a constitutively active Pka-C mutant subunit (Pka-C*) (Li et al., 1995) throughout the germline cells (Fig. 4C-E). This transgene, driven by a UAS promoter, has a point mutation that diminishes its ability to bind to PKA regulatory subunits; it therefore cannot be sequestered (Orellana and McKnight, 1992). We also used two lines that express GAL4 at different levels in the germline [*nos-GAL4-VP16* (VanDoren et al., 1998) and *NGT40* (Tracey et al., 2000)], to determine the relative dosage effects of active Pka-C. When Pka-C* was expressed in the germline, binucleate cells were produced at a frequency that correlated with the levels of GAL4 expression (*NGT40/+* < *nos-GAL4-VP16/+*; Table 2). Although both *NGT40* and *nos-GAL4-VP16* directed expression during the earliest stages of oogenesis, binucleate cells were only observed after approximately stage five or six. We observed a similar phenotype when *NGT40* was used to drive wild-type Pka-C instead of Pka-C*, although

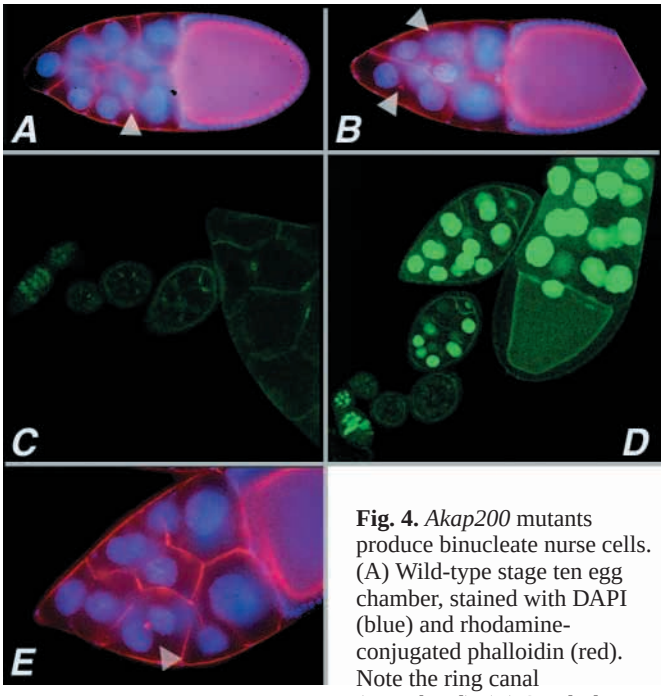

Fig. 4. *Akap200* mutants produce binucleate nurse cells. (A) Wild-type stage ten egg chamber, stained with DAPI (blue) and rhodamine-conjugated phalloidin (red). Note the ring canal (arrowhead). (B) Similarly staged *Akap200^{k07118}/Df(2L)N22-14* egg chamber, stained with DAPI and rhodamine phalloidin. Two binucleate nurse cells with ring canal remnants are indicated with arrowheads. (C-E) Expressing Pka-C throughout the germline with the GAL4/UAS system produces binucleate nurse cells. (C) Expression pattern of GAL4 line *NGT40*, visualized in *NGT40*/*UAS-GFPnls* ovaries. GAL4 expression was weak in this line, and was only detectable in germline cells in the germarium. (D) Expression pattern of GAL4 line *nos-GAL4-VP16*, visualized in *nos-GAL4-VP16*/*UAS-GFPnls* females. GAL4 expression was much stronger in this line, and highest in the germline cells in the germarium and after approximately stage seven. (E) Egg chambers from *NGT40/UAS-PkaC** females, stained with DAPI to visualize nuclei (blue) and rhodamine phalloidin (red). Note the binucleate nurse cell with a ring canal remnant (arrowhead). Binucleate cells with identical morphologies were observed in *nos-GAL4-VP16/UAS-Pka-C** ovaries. In C,D, the membrane and ring canal staining is due to bleed-through from the rhodamine-phalloidin channel.

fewer affected egg chambers resulted. Nevertheless, to ensure the analysis of all potential mutant egg chambers, we counted the frequency of binucleate cells at all stages between 2 and 11, resulting in binucleate cell frequencies of less than 10% for all egg chambers. No binucleate cells were observed with a peptide inhibitor of Pka-C (*UAS-PKIF*) (Kiger et al., 1999) or a mutant that fails to bind to Pka-C (*UAS-PKIG^{19,20}*) (Kiger et al., 1999), demonstrating that the binucleate cell phenotype was not due to GAL4 expression in the germline. Therefore, ectopic Pka-C activity disrupts the morphology of the germline cells.

Models of AKAP function have suggested two distinct roles for AKAPs: they either control the local activity of Pka-C by sequestering inhibitory subunits to specific subcellular locales, or they act as a scaffold, bringing PKA signaling components together. The first model predicts that increased Pka-C activity would either have no effect or enhance AKAP loss of function phenotypes, whereas in the scaffold model, increased Pka-C activity would overcome the loss of AKAP function. To distinguish between these roles of Akap200 in regulating actin morphology, we expressed Pka-C* in *Akap200* loss of function ovaries (Table 2). The frequency of multinucleate cells increased when Pka-C* was expressed in an *Akap200*/*Df(2L)N22-14* mutant background. These results are consistent with the hypothesis that Akap200 regulates the activity of the Pka-C subunit in the vicinity of subcortical actin in the nurse cells by localizing Pka-RII to these regions.

Akap200 expression levels affect ring canal size

Interestingly, we noticed during our studies that *Akap200* mutations also affected the size of the ring canals in the intact, mononucleate nurse cells. Tilney and colleagues measured ring canal size throughout oogenesis, and although they found variability because of the age of the ring canal, the size fell within a stereotypical range for a particular stage (Tilney et al., 1996). We noticed that in the strong *Akap200* alleles, the ring canals appeared to be larger and thinner than wild type. We stained ring canals with phalloidin, anti-Hts-RC and anti-phosphotyrosine, then measured the inner and outer ring canal diameter between stages seven and ten in mutant and wild-type egg chambers. We found that at each stage and with each

Table 2. Ectopic germline expression of Pka-C produces binucleate cells

Genotype†	Resulting expression pattern‡	% BNC§	*n*¶
Pka-C/+; nos-GAL4-VP16/+*	Constitutively active Pka-C throughout germline	7.3%	912
Pka-C/+; NGT40/+*	Constitutively active Pka-C throughout germline	3.1%	1616
Pka-C/+; NGT40/+	Normal Pka-C subunit throughout germline	1.2%	1608
UAS-PKIF/+; NGT40/+	Peptide inhibitor of Pka-C throughout germline	0%	749
UAS-PKIG^{19,20}/+; NGT40/+	Mutant peptide inhibitor of Pka-C throughout germline	0%	336
UAS-PKIG^{19,20}/+; nos-GAL4-VP16/+	Mutant peptide inhibitor of Pka-C throughout germline	0%	833
yw	Normal	0.1%	1760
yw/w; Pka-C Akap200^{k07118}/Df(2L)N22-14; +/+*	*Akap200* loss of function	19%	1103
yw/w; Pka-C Akap200^{k07118}/+; nos-GAL4-VP16/+*	Constitutively active Pka-C* throughout germline	4.5%	797
yw/w; Pka-C Akap200^{k07118}/Df(2L)N22-14; nos-GAL4-VP16/+*	*Akap200* loss of function with constitutively active Pka-C* throughout germline	30%	928

†Pka-C*: constitutively active Pka-C mutant subunit; *UAS-PKIF*: peptide inhibitor of Pka-C; *UAS-PKIG^{19,20}*: inactive mutant of *UAS-PKIF* (Kiger et al., 1999).
‡See Fig. 4C and D for relative levels of GAL4 expression in these lines.
§% BNC: Percent of egg chambers with at least one binucleate nurse cell.
¶*n*=total number of egg chambers scored (between stages two and eleven).

epitope, both the inner and outer rims were larger in the mutants (Table 3, Fig. 5). Although the ring canals were large and thin, phosphotyrosine, Hts-RC, Kelch and Filamin all appeared to localize normally to the mutant ring canals (data not shown). The *Akap200*$^{\Delta 7}$ allele also produced large, thin ring canals that were similar in size to *Akap200*^{k07118} ring canals (Table 3). By contrast, the weaker *Akap200*^{ix4} allele, which retained some Akap200 function, had an approximately normal ring canal size, even though it produced multinucleate nurse cells.

Because loss of *Akap200* function produced large ring canals, we hypothesized that the gain of *Akap200* function might result in smaller ring canals. We therefore overexpressed Akap200 in the nurse cells by crossing a GAL4-dependent 'enhancer piracy' line, *EP2254* (Rørth et al., 1998), which maps to the *Akap200* locus (FlyBase, 1999), to flies containing a germline-expressing GAL4 transgene (*nos-GAL4-VP16*). Ovaries from females carrying both *nos-GAL4-VP16* and *UAS-Akap200* produced egg chambers in which ring canals were smaller and thicker than wild type (Table 3 and Fig. 5). These females were fertile however, and the small ring canals did not interfere with the transfer of nurse cell contents to the oocyte (dumping; see Spradling, 1993). Although *nos-GAL4-VP16* was expressed at the earliest stages of oogenesis (Fig. 4D), we did not detect differences in ring canal morphology prior to stage four, nor did we observe other defects that could be correlated with altered germline stem cell function (i.e. extra or fewer divisions, changes in fate). Overexpressing Akap200 in the follicle cells resulted in ovaries that were indistinguishable from wild-type (data not shown).

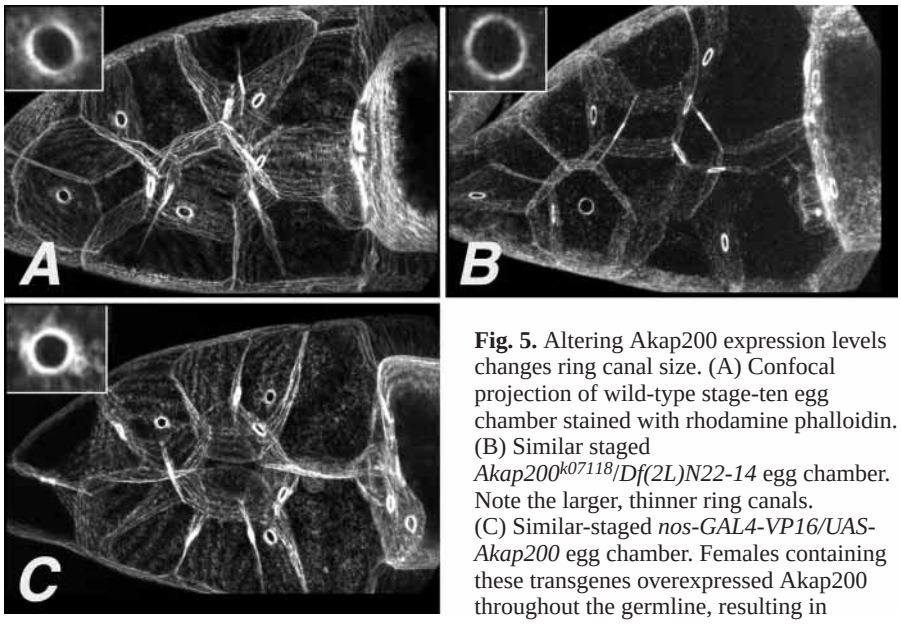

Fig. 5. Altering Akap200 expression levels changes ring canal size. (A) Confocal projection of wild-type stage-ten egg chamber stained with rhodamine phalloidin. (B) Similar staged *Akap200*^{k07118}*/Df(2L)N22-14* egg chamber. Note the larger, thinner ring canals. (C) Similar-staged *nos-GAL4-VP16/UAS-Akap200* egg chamber. Females containing these transgenes overexpressed Akap200 throughout the germline, resulting in smaller, thicker ring canals. Insets show a representative ring canal from each egg chamber, magnified a further four times.

Loss of *Akap200* function suppresses *Src64B* ring canal phenotype

Although many genes have been identified that alter ring canal function, altered ring canal size has only been observed in mutants of the non-receptor tyrosine kinases *Src64B* and *Tec29* (Dodson et al., 1998; Guarnieri et al., 1998; Roulier et al., 1998). In these mutants, ring canals are smaller, a phenotype opposite to that of the *Akap200* mutants. We examined ring canals in *Src64B* mutant ovaries when *Akap200* gene dose is reduced, to determine whether *Akap200* could antagonize *Src64B* in regulating ring canal size. The hypomorphic *Src64B*^{PI} allele (Dodson et al., 1998) is homozygous viable and produces ring canals that are smaller than wild type (Table 3 and Fig. 6A,B). *Akap200*^{k07118} acted as a dominant suppressor of the small ring canal size phenotype produced by these

Table 3. Changing Akap200 expression levels alters ring canal size

Genotype	Relative outer diameter‡	s.d.	*n*	Relative inner diameter‡	s.d.	*n*
normal§	1.00*	0.13	200	1.00*	0.14	105
Akap200^{k07118}*/Df(2L)N22-14*	1.14†	0.11	301	1.29†	0.20	217
nosG4/UAS-Akap200	0.89†	0.15	254	0.83†	0.13	140
w^{1118}	1.00†	0.11	160	1.00†	0.12	89
Src64B^{PI}*/Src64B*^{PI}	0.69*	0.07	165	0.69*	0.11	118
Akap200^{k07118}*/+; Src64B*^{PI}*/Src64B*^{PI}	0.92†	0.10	150	0.92†	0.14	87
Akap200^{k07118}*/Akap200*^{k07118}*; Src64B*^{PI}*/+*¶	1.09†	0.14	87	1.13†	0.17	58
w^{1118}*/+; Df(2L)N22-14/+*	1.00*	0.14	74	1.00*	0.09	48
Akap200^{k07118}*/Df(2L)N22-14*	1.24†	0.19	99	1.43†	0.16	62
Akap200$^{\Delta 7}$*/Df(2L)N22-14*	1.23†	0.13	81	1.38†	0.16	48
Akap200^{ix4}*/Df(2L)N22-14*	1.06††	0.21	83	1.17††	0.12	47

‡Outer diameters in mutants were normalized to outer diameters in control flies; inner diameters in mutants were normalized to inner diameters in controls. Differences between * and † were statistically significant using the Mann-Whitney U-test ($P<10^{-5}$); differences between * and †† were not statistically significant.

§These numbers include *daughterlessGAL4/UAS.Akap200* flies, *w*^{1118} flies, and *w*^{1118}*/+; Df(2L)N22-14/+* flies, all of which had similar ring canal diameters.

¶These flies are homozygous for *Akap200*^{k07118}, not hemizygous.

mutants. Females of genotype *Akap200*^{k07118}/+; *Src64B*^{PI}/*Src64B*^{PI} produce ring canals that are almost wild type in size and have near normal amounts of phosphotyrosine (Fig. 6C and Table 3). Reducing *Src64B* gene dose by half, however, failed to affect the size of the ring canals produced by *Akap200*^{k07118} mutants. Ring canals produced by *Akap200*^{k07118}/*Akap200*^{k07118}; *Src64B*^{PI}/+ were slightly smaller than those produced by *Akap200*^{k07118} mutants. These results suggest that *Src64B* and *Akap200* act antagonistically to regulate ring canal growth. Akap200 protein localization was not altered detectably in *Src64B*^{PI} mutant egg chambers (Fig. 6E), nor was *Src64B* localization changed visibly in *Akap200* mutant ovaries (data not shown). These observations suggest that post-translational mechanisms may be responsible for the genetic interaction.

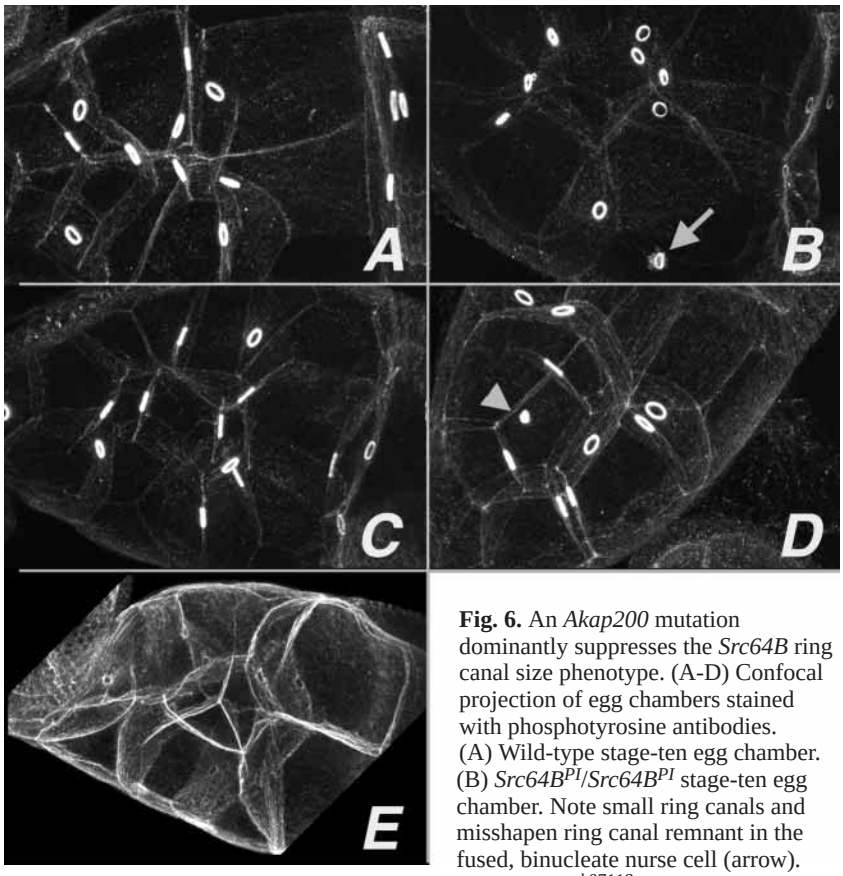

Fig. 6. An *Akap200* mutation dominantly suppresses the *Src64B* ring canal size phenotype. (A-D) Confocal projection of egg chambers stained with phosphotyrosine antibodies. (A) Wild-type stage-ten egg chamber. (B) *Src64B*^{PI}/*Src64B*^{PI} stage-ten egg chamber. Note small ring canals and misshapen ring canal remnant in the fused, binucleate nurse cell (arrow). (C) *Akap200*^{k07118}/+; *Src64B*^{PI}/*Src64B*^{PI} stage-ten egg chamber. The ring canals are near wild-type-sized. (D) *Akap200*^{k07118}/*Akap200*^{k07118}; *Src64B*^{PI}/+ stage-ten egg chamber. Ring canals are near normal sized; a ring canal remnant is indicated with an arrowhead. (E) *Src64B*^{PI}/*Src64B*^{PI} early stage-ten egg chamber stained with Akap200 antiserum. Except for the small ring canals, Akap200 localization in these mutants appears normal.

DISCUSSION

In this study, we analyzed the role of PKA anchoring in a developmental context. We showed that Akap200 and Pka-RII colocalize to membranes and ring canals during oogenesis and that loss of *Akap200* function diminishes Pka-RII membrane localization. Moreover, loss of *Akap200* function affects the structural integrity of the germline-derived cells. Expressing a mutant PKA catalytic subunit, incapable of associating with regulatory subunits and therefore incapable of being properly sequestered, produces similar phenotypes to loss of *Akap200* function. *Akap200* mutations result in large, thin ring canals; conversely, Akap200 overexpression in the germline yields smaller, thicker ring canals. Finally, loss of *Akap200* function suppresses the small ring canal phenotype produced by *Src64B* mutants.

Localized PKA activity and cytoarchitecture

Our results provide in vivo support of a model for regulating Pka-C activity by subcellular sequestration (Fig. 7). We propose that Akap200 (green rectangles) in the vicinity of ring canals and nurse cell membranes anchors Pka-RII (dark blue rectangles) to these same subcellular regions. In turn, Pka-RII binds and inactivates Pka-C (pink rectangles); the Akap200-Pka-RII complex may be thought of as a ‘sponge’ for active Pka-C subunit. Some, if not a majority, of the Pka-C subunits in the germline are sequestered in the vicinity of membranes as well and may result from associations with the Akap200-PkaRII complex (Lane and Kalderon, 1995). Several different cytoskeleton-associated proteins (yellow circles and orange blobs) that regulate the morphology of filamentous actin (red lines) might be regulated by Pka-C phosphorylation, and the combination of activities of these other proteins and Pka-C ensures that the actin filaments are properly assembled and their morphology maintained.

In this model, Akap200 could have two roles. First, by binding to other proteins besides Pka-RII, Akap200 may act as a scaffold that brings substrates and other regulatory proteins to the membrane, ensuring rapid, local responses to PKA activation. Alternatively, by interacting with the inhibitory regulatory subunits, Akap200 may act as a ‘sponge’ to ensure that concentrations of active Pka-C subunit are precisely controlled at the subcellular level. Our results, together with studies of *Pka-C* loss-of-function in the ovary, suggest that the precise concentration of Pka-C activity at the membrane is critical to regulate the morphology of actin structures in egg chambers, and that one of the functions of Akap200 is to control this activity. In Pka-C loss of function mutants, some of the cytoskeleton-associated proteins that require Pka-C phosphorylation (orange blobs in Fig. 7) may fail to become active. As a result, local instabilities in subcortical actin filaments may provide weak points for rapid disassembly of the remaining filaments, ultimately producing a binucleate nurse cell. In *Akap200* mutants, Pka-RII cannot be sequestered; the regulatory ‘sponge’ discussed above therefore does not form properly. Similarly, when a mutant Pka-C incapable of being properly sequestered is expressed in the nurse cells, it does not

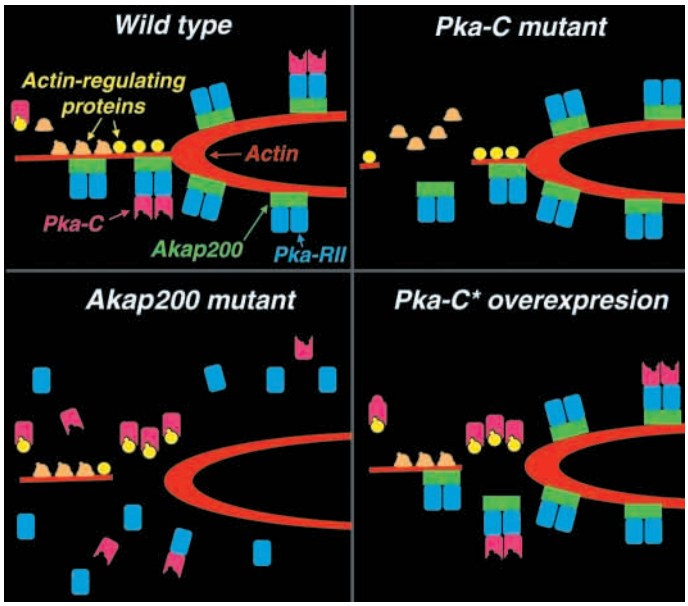

Fig. 7. Model for localized regulation of Protein kinase A activity by *Akap200*. See Discussion for details. Red depicts filamentous actin in subcortical regions of nurse cells and ring canals, green symbolizes Akap200, dark blue is Pka-RII, pink is Pka-C; yellow circles and orange blobs are other proteins that regulate actin filament polymerization or function. Phosphorylation by Pka-C inhibits the actin-regulating activity of the yellow circle proteins in this diagram, whereas Pka-C phosphorylation is required for the orange blob proteins to regulate actin function properly. Pka-C* is a mutant isoform of catalytic subunit that is incapable of binding to Pka-RII.

matter that AKAP200/Pka-RII 'sponge' is localized appropriately. These two changes produce higher local concentrations of active Pka-C in the vicinity of subcortical actin and ring canals, which in turn phosphorylate other actin-associated proteins (yellow circles in Fig. 7) and modify their activity inappropriately. Although a different set of cytoskeleton-associated proteins may be misregulated by ectopic Pka-C activity than loss of Pka-C activity, the end result of local destabilization of actin filaments and nurse cell fusion could be the same. This hypothesis predicts that loss of Pka-RII function would also result in ectopic, active Pka-C and would produce a similar phenotype. Indeed, nurse cell fusions with very similar morphology were reported in Pka-RII mutant ovaries (Park et al., 2000). Finally, one of the predictions of a scaffold model is that expressing Pka-C* throughout the germline cells might be expected to overcome the lack of a scaffold in *Akap200* mutants. We found, however, that expressing Pka-C* in *Akap200* mutants exacerbated the frequency of binucleate cell production. These results support the hypothesis that the amount of active Pka-C is regulated locally in the ovary and argue against the need for Akap200 to bring Pka-C to the vicinity of substrate molecules. Nevertheless, these two mechanisms are not mutually exclusive, and Pka-C regulation by Akap200 in these cells could be complex. The identities of the hypothesized actin-associated proteins remain to be determined. One intriguing candidate is the p21-PAK – a regulator of actin filament dynamics via LIM kinase and cofilin, shown recently to be down-regulated by Pka-C phosphorylation (Howe and Juliano, 2000). Other likely candidates include those uncovered in a screen for mutations that produce binucleate cells (Jackson and Berg, 1999).

Mechanism of Akap200 action

Loss of *Akap200* does not completely eliminate all Pka-RII membrane localization, possibly because even our strongest alleles, which eliminate all detectable Akap200 protein, may not be null. Other proteins besides Akap200 are also likely to contribute to Pka-RII sequestration in the vicinity of the membranes. In mammalian cells, the widely expressed AKAP75/79/150 sequesters Pka-RII subunit to membranes in transfected HEK293 cells (Li et al., 1996), and ezrin, an actin-binding protein, is an AKAP in gastric mucosal cells (Dransfield et al., 1997). Neither of these proteins can be responsible for the residual Pka-RII membrane staining in Akap200 mutants however, because no obvious homologs to either AKAP75/79/150 or ezrin exist in the fly genome (FlyBase, 1999). In fact, the overall lack of strong sequence homology among AKAPs hinders our ability to identify homologs by database searching. Other Pka-RII binding proteins that may be present in the fly ovary must therefore be identified using other methods.

The subcellular localization of endogenous Akap200 in ovaries is consistent with previous studies showing that the myristoylation site within the MARCKS domain is responsible for Akap200 membrane association in transfected Schneider cells (Rossi et al., 1999). Our studies do not distinguish whether the localization in the ovary results from direct membrane association via the myristoylation modification, or from associations with subcortical actin via the actin-binding motif found within MARCKS domain proteins (Aderem, 1992). The localization of Akap200 on the outer rim of ring canals could be explained by direct interactions with the filamentous actin found on the inner rim of the ring canal, or by embedding Akap200 into the plasma membrane near the outer rim via its myristoylation motif. Interestingly, we did not detect any Akap200 associated with other filamentous actin-containing structures in the ovary, such as the spectrosome, the fusome or the actin fibers that anchor nurse cell nuclei during dumping (see Robinson and Cooley, 1997). This observation raises the possibility that Akap200 does not bind to ovarian actin filaments directly; rather, the myristoylation domain may embed Akap200 in the membrane near subcortical actin and ring canals. Alternatively, the filamentous actin-binding activity of Akap200 may be regulated, such that Akap200 is capable of binding only to subsets of actin filaments in the ovary. This regulation may be mediated by Protein kinase C or CAM kinase modifications in the MARCKS domain (Aderem, 1992), or phosphorylation elsewhere in the protein by non-receptor tyrosine kinases. Further experimentation is needed to distinguish among these possibilities.

Effects on ring canal morphology

One surprising phenotype observed in Akap200 mutants was that ring canals were larger. The mechanism by which ring canal size is regulated during oogenesis is not yet clear. Mutations in two non-receptor tyrosine kinase genes (*Src64B* and *Tec29*) make smaller ring canals and are important for regulating ring canal size (Dodson et al., 1998; Guarnieri et al., 1998; Roulier et al., 1998). Interestingly, these mutations also produce binucleate cells; however, abnormally sized ring canals have not

been described in other mutants that make multinucleate cells (such as *chickadee* or *cappuccino*) (Manseau et al., 1996). Unexpectedly, we found that changing Akap200 levels in the germline influenced the size of the ring canals. We envision two general mechanisms that could produce changes in ring canal size: defective architecture or defective temporal control. First, defective assembly of ring canal components could produce ring canals that cannot respond to structural stresses appropriately. For example, in *Akap200* mutants, a component of the ring canal may be missing or misregulated, resulting in a complex that is not tightly held together. The diameter of the ring canal could therefore expand abnormally, producing larger, thinner ring canals. Conversely, the smaller, thicker ring canals observed when Akap200 was overexpressed and in *DSr64B* mutants could be produced if the entire ring canal complex contracted abnormally as a result of a misregulated or missing component. The second possibility is that stage-specific assembly of the entire ring canal complex may be accelerated (or arrested), resulting in larger (or smaller) ring canals that are inappropriately sized for the stage of the egg chamber. In other words, the large ring canals seen in *Akap200* mutants could be explained by hypothesizing that stage nine nurse cells 'think' they should be making stage ten-sized ring canals. Because *Src64B*, *Tec29* and *Akap200* are all linked to the actin cytoskeleton, we favor the first hypothesis, but we cannot discount the second possibility because the order of assembly and the regulation of ring canal growth are incompletely understood processes.

Nevertheless, the *Akap200* gene product likely contributes to altered ring canal size in two non-exclusive ways. First, proteins regulating ring canal growth and morphology could affect the functional activity of Akap200. In addition, *Akap200* could influence the expression levels, subcellular localization or functional activity of proteins associated with ring canals.

We tested the first possibility by examining Akap200 localization in mutants that affect ring canal size and structure. However, no changes in Akap200 expression levels or localization were detected in $Src64B^{PI}$ (Fig. 6E) or in hts^{1} mutants (data not shown). Nevertheless, future analyses may demonstrate that the localization pattern of Akap200 in other mutants is directly regulated by a gene that regulates ring canal size. Alternatively, *Akap200* may regulate the localization or function of a protein that is assembled into ring canals. We tested this second possibility by examining ring canal structure in *Akap200* mutants. We could not detect significant differences in the expression levels or subcellular localization of known ring canal-associated proteins or markers (such as phalloidin, Hts-RC, anti-phosphotyrosine, Kelch, Filamin and Src64B). Critical gene products that do undergo such a change may exist, but these remain hitherto unidentified. Furthermore, covalent modifications that change the function of the protein but not the expression levels or localization would not be detected by our assays and would require biochemical experiments to detect such modifications. The analysis of other ring canal-associated mutants and their interaction with *Akap200* may eventually provide insights into the mechanism of ring canal growth.

How do the ring canals fit into our model explaining the nurse cell fusions? The larger, thinner ring canals could be a manifestation of weakened supporting elements in the vicinity of the ring canal that result from ectopic active Pka-C-mediated phosphorylation of the other cytoskeleton-associated proteins. This structural weakening could provide an opportunity for the ring canal to expand and ultimately break, resulting in a retraction of membrane from the ring canal and freeing of a ring canal remnant.

Alternatively, the increased ring canal size may be unrelated to the formation of multinucleate cells; rather, a second mechanism prevents fusion of the nurse cells. This hypothesis raises the intriguing possibility that the two domains of Akap200 provide different functions: the MARCKS domain may regulate ring canal size, whereas the Pka-RII binding region may be involved in preserving the cytoarchitecture of the cells. In support of this second hypothesis, multinucleate cells with approximately normal-sized ring canals are produced in the hypomorphic $Akap200^{ix4}$ allele, in other PKA signaling- and cytoskeleton-associated mutants, and by overexpressing Pka-C in the germline cells. Thus, the large ring canal phenotype may be separable from the nurse-cell fusion phenotype. Although other binding partners for Akap200 are not currently known, this protein may bring together both PKA and PKC signaling pathways at or near the actin cytoskeleton (Li et al., 1999). One of these other pathways may be linked to regulating ring canal size, but not the formation of multinucleate cells.

Sequestering PKA: implications for function

The importance of PKA localization has been the subject of investigation and speculation for several years. For example, PKA localization is important in regulating the activity of ion channels in the context of cultured cells (Ali et al., 1998; Fraser et al., 1998; Gao et al., 1997) or Xenopus oocytes (Ali et al., 1998). In contrast, other studies found that localization of Pka-RII by AKAPs may not be required for calcium channel modulation in skeletal muscle (Burton et al., 1997) or for mouse sperm motility (Burton et al., 1999). Our analyses of *Akap200* mutants provide evidence that PKA localization is important for regulating the function of specialized actin structures during oogenesis in *Drosophila*. The analysis of AKAP mutant phenotypes in model organisms may provide the tissue- and cell-specific context necessary to detect subtle phenotypes that have profound consequences on cellular functions.

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REFERENCES

Aderem, A. (1992). The MARCKS brothers: a family of protein kinase C substrates. *Cell* **71**, 713-716.

Ali, S., Chen, X., Lu, M., Xu, J. Z., Lerea, K. M., Hebert, S. C. and Wang, W. H. (1998). The A kinase anchoring protein is required for mediating the effect of protein kinase A on ROMK1 channels. *Proc. Natl. Acad. Sci. USA* **95**, 10274-10278.

Burton, K. A., Johnson, B. D., Hausken, Z. E., Westenbroek, R. E., Idzerda, R. L., Scheuer, T., Scott, J. D., Catterall, W. A. and McKnight, G. S. (1997). Type II regulatory subunits are not required for the anchoring-

dependent modulation of Ca2+ channel activity by cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **94**, 11067-11072.

Burton, K. A., Treash-Osio, B., Muller, C. H., Dunphy, E. L. and McKnight, G. S. (1999). Deletion of type IIalpha regulatory subunit delocalizes protein kinase A in mouse sperm without affecting motility or fertilization. *J. Biol. Chem.* **274**, 24131-24136.

Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D., Bishop, S. M., Acott, T. S., Brennan, R. G. and Scott, J. D. (1991). Interaction of the regulatory subunit (RII) of cAMP-dependent protein kinase with RII-anchoring proteins occurs through an amphipathic helix binding motif. *J. Biol. Chem.* **266**, 14188-14192.

Colledge, M. and Scott, J. D. (1999). AKAPs: from structure to function. *Trends Cell Biol.* **9**, 216-221.

Cooley, L. (1998). *Drosophila* ring canal growth requires Src and Tec Kinases. *Cell* **93**, 913-915.

Crittenden, J. R., Skoulakis, M. C., Han, K., Kalderon, D. and Davis, R. L. (1998). Tripartite mushroom body architecture revealed by antigenic markers. *Learn. Mem.* **5**, 38-51.

Dodson, G. S., Guarnieri, D. J. and Simon, M. A. (1998). *Src64* is required for ovarian ring canal morphogenesis during *Drosophila* oogenesis. *Development* **125**, 2883-2892.

Dransfield, D. T., Bradford, A. J., Smith, J., Martin, M., Roy, C., Mangeat, P. H. and Goldenring, J. R. (1997). Ezrin is a cyclic AMP-dependent protein kinase anchoring protein. *EMBO J.* **16**, 35-43.

FlyBase (1999). The FlyBase database of the *Drosophila* genome projects and community literature. *Nucleic Acids Res.* **27**, 85-88. http://flybase.bio.indiana.edu/.

Fraser, I. D. C., Tavalin, S. J., Lester, L. B., Langeber, L. K., Westphal, A. M., Dean, R. A., Marrion, N. V. and Scott, J. D. (1998). A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-mediated membrane events. *EMBO J.* **17**, 2261-2272.

Gao, T., Yatani, A., Dell'Acqua, M. L., Sako, H., Green, S. A., Dascal, N., Scott, J. D. and Hosey, M. M. (1997). cAMP-dependent regulation of cardiac L-type Ca+2 channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron* **19**, 185-196.

Gross, R. E., Bagchi, S., Lu, X. and Rubin, C. S. (1990). Cloning, characterization, and expression of the gene for the catalytic subunit of cAMP-dependent protein kinase in *Caenorhabditis elegans*. Identification of highly conserved and unique isoforms generated by alternative splicing. *J. Biol. Chem.* **265**, 6896-6907.

Guarnieri, D. J., Dodson, G. S. and Simon, M. A. (1998). *Src64* regulates the localization of a Tec family kinase for *Drosophila* ring canal growth. *Mol. Cell* **1**, 831-840.

Han, J. D., Baker, N. E. and Rubin, C. S. (1997). Molecular characterization of a novel A kinase anchor protein from Drosophila melanogaster. *J. Biol. Chem.* **272**, 26611-26619.

Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J., Taylor, S. S., Scott, J. D. and Korsmeyer, S. J. (1999). Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. *Mol. Cell* **3**, 413-422.

Howe, A. K. and Juliano, R. L. (2000). Regulation of anchorage-dependent signal transduction by Protein kinase A and p21-activated kinase. *Nat. Cell Biol.* **2**, 593-600.

Huang, L. J., Durick, K., Weiner, J. A., Chun, J. and Taylor, S. S. (1997). Identification of a novel protein kinase A anchoring protein that binds both type I and type II regulatory subunits. *J. Biol. Chem.* **272**, 8057-8064.

Jackson, S. M. and Berg, C. A. (1999). Soma-to-germline interactions during *Drosophila* oogenesis are influenced by dose-sensitive interactions between *cut* and the genes *cappuccino*, *ovarian tumor* and *agnostic*. *Genetics* **153**, 289-303.

Jackson, S. M. and Blochlinger, K. (1997). *Cut* interacts with *Notch* and *protein kinase A* to regulate follicle formation and to maintain germline cyst integrity during *Drosophila* oogenesis. *Development* **124**, 3663-3672.

Johnson, B. D., Scheuer, T. and Catterall, W. A. (1994). Voltage-dependent potentiation of L-type Ca^{+2} channels in skeletal muscle cells requires anchored cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **91**, 11492-11496.

Jowett, T. (1998). Preparation of nucleic acids. In *Drosophila, A Practical Approach (2E)* (ed. D. B. Roberts), pp. 347-371. Oxford, UK: Oxford University Press.

Kalderon, D. and Rubin, G. M. (1988). Isolation and characterization of *Drosophila* cAMP-dependent protein kinase genes. *Genes Dev.* **2**, 1539-1556.

Kiger, J. A., Eklund, J. L., Younger, S. H. and O'Kane, C. J. (1999). Transgenic inhibitors identify two roles for protein kinase A in *Drosophila* development. *Genetics* **152**, 281-290.

Klussmann, E., Maric, K., Wiesner, B., Beyermann, M. and Rosenthal, W. (1999). Protein kinase A anchoring proteins are required for vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principle cells. *J. Biol. Chem.* **274**, 4934-4938.

Lane, M. E. and Kalderon, D. (1995). Localization and functions of Protein Kinase A during *Drosophila* oogenesis. *Mech. Dev.* **49**, 191-200.

Li, W., Ohlmeyer, J. T., Lane, M. E. and Kalderon, D. (1995). Function of Protein kinase A in hedgehog signal transduction and *Drosophila* imaginal disc development. *Cell* **80**, 553-562.

Li, Y., Ndubuka, C. and Rubin, C. S. (1996). A kinase anchor protein 75 targets regulatory (RII) subunits of cAMP-dependent protein kinase II to the cortical actin cytoskeleton in non-neuronal cells. *J. Biol. Chem.* **271**, 16862-16869.

Li, Z., Rossi, E., Hoheisel, J., Kalderon, D. and Rubin, C. (1999). Generation of a novel A kinase anchoring protein and a myristoyalted alanine-rich C kinase substrate-linked analog from a single gene. *J. Biol. Chem.* **274**, 27191-27200.

Lu, X. Y., Gross, R. E., Bagchi, S. and Rubin, C. S. (1990). Cloning, structure, and expression of the gene for a novel regulatory subunit of cAMP-dependent protein kinase in *Caenorhabditis elegans*. *J. Biol. Chem.* **265**, 3293-3303.

Manseau, L., Calley, J. and Phan, H. (1996). Profilin is required for posterior patterning of the *Drosophila* oocyte. *Development* **122**, 2109-2116.

Mochly-Rosen, D. (1995). Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science* **268**, 247-251.

Orellana, S. A. and McKnight, G. S. (1992). Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity. *Proc. Natl. Acad. Sci. USA* **89**, 4726-4730.

Park, S. K., Sedore, S. A., Cronmiller, C. and Hirsh, J. (2000). Type II cAMP-dependent protein kinase-deficient *Drosophila* are viable but show developmental, circadian, and drug response phenotypes. *J. Biol. Chem.* **275**, 20588-20596.

Robinson, D. N., Cant, K. and Cooley, L. (1994). Morphogenesis of *Drosophila* ovarian ring canals. *Development* **120**, 2015-2025.

Robinson, D. N. and Cooley, L. (1997). Genetic analysis of the actin cytoskeleton in the *Drosophila* ovary. *Ann. Rev. Cell Dev. Biol.* **13**, 147-170.

Rørth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G. M., Weigmann, K., Milan, M., Benes, V., Ansorge, W. et al. (1998). Systematic gain-of-function genetics in *Drosophila*. *Development* **125**, 1049-1057.

Rosenmund, C., Carr, D. W., Bergeson, S. E., Nilaver, G., Scott, J. D. and Westbrook, G. L. (1994). Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. *Nature* **368**, 853-856.

Rossi, E. A., Li, Z., Feng, H. and Rubin, C. S. (1999). Characterization of the targeting, binding and phosphorylation site domains of an A kinase anchor protein and a myristoylated alanine-rich C kinase substrate-like analog that are encoded by a single gene. *J. Biol. Chem.* **274**, 27201-27210.

Roulier, E. M., Panzer, S. and Beckendorf, S. K. (1998). The nonreceptor tyrosine kinase Tec29A is required during *Drosophila* embryogenesis and interacts with Src64B in ring canal development. *Mol. Cell* **1**, 819-829.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Lab Manual*. New York, USA: Cold Spring Harbor Laboratory Press.

Scott, J. D. and McCartney, S. (1994). Localization of A-kinase through anchoring proteins. *Mol. Endocrinol.* **8**, 5-11.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In *The Development of* Drosophila Melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 1-69. Plainview, New York, USA: Cold Spring Harbor Laboratory Press.

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S. and Rubin, G. M. (1999). The Berkeley *Drosophila* genome project gene disruption project: single *P* element insertions mutating 25% of vital *Drosophila* genes. *Genetics* **153**, 135-177.

Tilney, L. G., Tilney, M. S. and Guild, G. M. (1996). Formation of actin filament bundles in the ring canals of developing *Drosophila* follicles. *J. Cell Biol.* **133**, 61-74.

Tracey, W. D., Jr, Ning, X., Klingler, M., Kramer, S. G. and Gergen, J. P. (2000). Quantitative analysis of gene function in the *Drosophila* embryo. *Genetics* **154**, 273-284.

VanDoren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr. Biol.* **8**, 243-246.

Wang, Z. W. and Kotlikoff, M. I. (1996). Activation of K_{Ca} channels in airway smooth muscle cells by endogenous protein kinase A. *Am. J. Physiol.* **271**, L100-L105.