Production of *gurken* in the nurse cells is sufficient for axis determination in the *Drosophila* oocyte

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Accepted 8 March 2005

Development 132, 2345-2353
Published by The Company of Biologists 2005
doi:10.1242/dev.01820

Summary

The asymmetric localization of *gurken* mRNA and protein in the developing *Drosophila* oocyte defines both the anteroposterior and dorsoventral axes of the future embryo. Understanding the origin of these asymmetries requires knowledge of the source of *gurken* transcripts. During oogenesis most transcripts in the oocyte are produced by the associated nurse cells, but it has been proposed that *gurken* is an exceptional oocyte-derived transcript. Using a novel application of a standard mitotic recombination technique, we generated mosaic egg chambers in which the nurse cells, but not the oocyte, could produce *gurken*. Gurken was properly localized in these mosaics and oocyte axial polarity was established normally, indicating that the nurse cells synthesize *gurken* and that their contribution is sufficient for Gurken function. Our data demonstrate the existence of a mechanism for transport of *gurken* from the nurse cells and its subsequent localization within the oocyte.

Key words: *Drosophila*, Polarity, *gurken*, Genetic mosaic

Introduction

In *Drosophila*, as in many invertebrates and some vertebrates, the fundamental asymmetries that define the embryonic body axes are established during oogenesis through the localization of maternally contributed cytoplasmic determinants within the egg. The restricted distribution of these factors is often achieved through localization of maternal transcripts within the developing oocyte, and differential translational regulation of the localized and unlocalized mRNAs. Polarization of both the anteroposterior (AP) and dorsoventral (DV) axes of the *Drosophila* embryo requires the precise localization of a single cytoplasmic determinant: the product of the *gurken* (*grk*) gene (Cooperstock and Lipshitz, 2001; Roth, 2003; Van Buskirk and Schüpbach, 1999). While the molecular mechanism of Grk function and the requirement for its proper localization have been well established, elucidation of the mechanism(s) regulating Grk localization requires an understanding of the source of *grk* transcripts. To address this issue, we have taken a genetic approach to investigate the site of *grk* production within the *Drosophila* egg chamber.

Each *Drosophila* oocyte develops within a cyst of 16 germline cells, which is surrounded by an epithelium of somatic follicle cells to form a developmental unit called an egg chamber (Spradling, 1993). Each 16-cell cyst is derived from a single cell, the cystoblast, through four synchronized mitoses. Cytokinesis is incomplete during these divisions, forming a stereotypic pattern of cytoplasmic bridges, called ring canals, between the cells of the cyst (see Fig. 1A). One of the cells with four ring canals develops as the oocyte, and comes to occupy the posteriormost position in the cyst, while the other 15 cells become nurse cells, which supply essential components to the oocyte through the ring canals. The AP axis of the egg chamber is established when the oocyte adopts its posterior position within the cyst. The final AP polarity of the oocyte itself is generated during mid-oogenesis through a localized signal from the oocyte to the overlying follicle cells. In later stages, another localized signaling event between the oocyte and follicle cells defines the DV axis of the egg chamber (Huynh and St Johnston, 2004; Van Buskirk and Schüpbach, 1999).

In each of these oocyte-patterning steps, the signal from the oocyte is encoded by the *grk* gene. The Grk protein, which is related to transforming growth factor-α, functions as a spatially restricted ligand secreted by the oocyte to activate the *Drosophila* epidermal growth factor receptor (Egfr) in the follicle cells (Neuman-Silberberg and Schüpbach, 1993), resulting in localized Egfr signaling, which generates axial polarity. In early oogenesis, *grk* mRNA is localized to the posterior cortex of the oocyte, adjacent to the oocyte nucleus. As the oocyte is small at this stage, the Grk signal is restricted to the overlying follicle cells at the posterior of the egg chamber and induces them to adopt a posterior fate, thus distinguishing them from anterior cells and establishing the AP polarity of the follicular epithelium (Gonzalez-Reyes et al., 1995; Gonzalez-Reyes and St Johnston, 1998; Roth et al., 1995). The posterior follicle cells in turn provide a signal that induces a reorganization of the oocyte cytoskeleton and thereby confers appropriate polarity on the oocyte AP axis. One consequence of this reorganization is the microtubule-dependent migration of the oocyte nucleus to the anterior cortex of the oocyte, where its position along the oocyte circumference provides the first detectable asymmetry along the DV axis. Both *grk* mRNA and protein remain closely associated with the oocyte nucleus, and at this new anterior

position they provide a localized signal to a second population of follicle cells, resulting in the induction of dorsal follicle cell fates and establishing the DV axis of the egg chamber.

Proper localization of Grk is crucial for the generation of axial polarity. Mutations in the *maelstrom* gene alter *grk* mRNA localization in early stages and result in defective posterior follicle cell fate determination (Clegg et al., 1997). In ovaries overexpressing *grk* or lacking the function of *squid* (*sqd*) or *fs(1)K10*, *grk* is not restricted dorsally but is instead distributed throughout the anterior cortex of the oocyte, resulting in expanded induction of dorsal follicle cell fates (Neuman-Silberberg and Schüpbach, 1993). The asymmetric localization of *grk* within the oocyte requires the association of *grk* transcripts with hnRNPs and the activity of the microtubule motor proteins Dynein and Kinesin (Brendza et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002; MacDougall et al., 2003). However, how these factors function to achieve the unique dorsal anterior localization of *grk* is not well understood.

The close association of *grk* mRNA and protein with the oocyte nucleus has led to the proposal that anchoring of the message upon export from the oocyte nucleus mediates or contributes to its localization (Goodrich et al., 2004; Norvell et al., 1999; Palacios and St Johnston, 2001; Saunders and Cohen, 1999). This model has been supported by evidence suggesting that *grk* is transcribed primarily or exclusively in the oocyte nucleus, derived from experiments designed to exclude the possibility of transport from the nurse cells to the oocyte (Saunders and Cohen, 1999). Such a mechanism would require that *grk* be a rare gene transcribed in the oocyte nucleus, which is otherwise transcriptionally quiescent and arrested in meiotic prophase (King and Burnett, 1959; Spradling, 1993). Alternatively, *grk* may be transcribed in the nurse cells and transported to the oocyte, as are transcripts encoding other spatially restricted determinants, such as *bicoid* and *oskar* (*osk*), found at the anterior and posterior poles, respectively (Johnstone and Lasko, 2001; Lipshitz and Smibert, 2000; Palacios and St Johnston, 2001). Production of *grk* in the nurse cells would require an alternative model for localization, as a localized synthesis and retention mechanism would not apply to nurse-cell-derived transcripts. For example, microtubule-based transport has been shown to function in *grk* localization within the oocyte. However, since the existence of a localization mechanism within the oocyte could equally be proposed to localize nurse-cell-derived messages or to maintain localization of oocyte-derived messages, this observation does not address the source of *grk* transcripts.

Using a novel application of a standard genetic technique to address this question, we generated egg chambers with mosaic germline cysts in which the oocyte lacked the ability to produce *grk* but the nurse cells retained *grk* function. This genetic approach provided a stringent functional test for *grk* production: if *grk* were produced exclusively in the oocyte nucleus, then mosaic egg chambers with a mutant oocyte would be predicted to exhibit patterning defects. Our results demonstrate that the nurse cells produce functional *grk*, and that their contribution is sufficient for proper Grk localization within the oocyte and establishment of the oocyte AP and DV axes. While our data do not exclude the oocyte nucleus as a potential additional source of *grk* transcripts, any such contribution is not required for axis determination. Our findings imply the existence of a mechanism for transport of *grk* from the nurse cells and its subsequent localization within the oocyte.

Materials and methods

Fly stocks

For mosaic analysis of *grk* function, we used *w; P{neoFRT}40A* (Xu and Rubin, 1993) and *w; grk^{2B6} cn bw/ CyO* (gift from T. Schüpbach) to generate *grk^{2B6} P{neoFRT}40A*. The *grk^{2B6}* null allele, which contains a 442 bp deletion that removes the transcriptional start site and part of the promoter, produces no *grk* RNA or protein and is the strongest existing *grk* allele (Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1994; Thio et al., 2000). Clones marked by the absence of nuclear localized green fluorescent protein (GFPnls) were generated using *y w P{hsFLP}122*; *P{Ubi-GFPnls}2-1 P{neoFRT}40A* (provided by S. Luschnig). Strains for the *lacO* marker system were *w*; *P{Hsp83-GFP::lacI}* and *w; P{lacO^{256x}}* (Vazquez et al., 2001). We mobilized a third chromosome insert of *w; P{lacO^{256x}}* to generate an insert on 2L, then generated *w; grk^{2B6} P{lacO^{256x}}2L P{neoFRT}40A*. For visualization of *mirr* expression, we used the *P{lacW}l(2)mirr^{6D1}* enhancer trap (gift from D. Morisato).

Production of germline mosaic egg chambers

Mosaic ovaries with the nuclear GFP clone marker were produced by FLP-FRT-mediated mitotic recombination (Xu and Rubin, 1993) in females of the following two genotypes: *y w P{hsFLP}122; grk^{2B6} P{neoFRT}40A/P{Ubi-GFPnls}2-1 P{neoFRT}40A* and *y w P{hsFLP}122; grk^{2B6} P{neoFRT}40A/P{Ubi-GFPnls}2-1 P{neoFRT}40A; P{lacW}l(2)mirr^{6D1}*. To induce recombination, pupae were heatshocked in a water bath for 1 hour at 37°C on each of three consecutive days beginning 2 days after puparium formation. The vials were maintained at 25°C at all other times, and adult ovaries were harvested 10 days after the first heatshock.

To generate ovaries mosaic for the *lacO* transgene, recombination was induced as above in females of the following genotype: *y w P{hsFLP}122; grk^{2B6} P{lacO^{256x}}2L P{neoFRT}40A/P{NM}31E P{neoFRT}40A*; *P{Hsp83-GFP::lacI}*. In addition, females were heatshocked at 37°C for 20 minutes, 7 hours before dissection to induce expression of GFP::LacI. To confirm that the number of foci of GFP fluorescence observed in the oocyte nucleus corresponds to the number of copies of the *P{lacO^{256x}}* transgene present, we induced GFP::LacI expression in non-mosaic females either homozygous or heterozygous for a transgene-containing chromosome. Multiple fluorescent foci are readily detected in all nurse cell nuclei, which are polyploid, but individual foci in the oocyte nucleus are more difficult to detect. In females with two copies of the *lacO* transgene, two foci were detected in 6/49 stage-8-9 egg chambers, and the remainder exhibited either one focus (8/49) or no foci (35/49). A single focus of GFP fluorescence was detected in the oocyte in 4/23 stage-8-9 egg chambers from females with a single copy of the transgene; importantly, two foci were never observed, indicating that the presence of two foci is diagnostic for homozygosity of the transgene-containing chromosome. Because the inefficiency of detection of the *lacO* transgene in the oocyte nucleus prevented the unambiguous diagnosis of its absence, we placed the *lacO* transgene in *cis* to the *grk^{2B6}* allele to allow positive identification of homozygous mutant oocytes by the presence of two foci in the nucleus (see Fig. 1C). Foci were undetectable in nearly all egg chambers after stage 9, precluding the use of this marker for analysis of DV patterning.

Immunohistochemistry

Ovaries were dissected in phosphate-buffered saline (PBS) and fixed at room temperature for 20 minutes in 5% formaldehyde (EM grade; Polysciences, Inc), in PBS with 1% NP40, saturated with heptane,

then washed three times for 10 minutes in PBS with 0.3% Triton-X100 (PBST). Ovaries were further permeabilized by incubating at room temperature for 1 hour in PBS with 1% Triton-X100, then blocked for 1 hour in PBST with 1% bovine serum albumin (BSA). Ovaries were then incubated overnight at 4°C in PBST with a 1:100 dilution of primary monoclonal antibody [anti-Grk, MAb1D12, or anti-β-galactosidase, MAb40-1a, concentrated, Developmental Studies Hybridoma Bank; anti-Broad Core (BR-C) MAb25E9, supernatant, gift of Greg Guild], washed three times for 20 minutes in PBST, incubated for 1 hour in PBST with 1% BSA, then incubated for 90 minutes with goat anti-mouse AlexaFluor^{568nm} (1:1000, Molecular Probes) in PBST. Samples were washed three times for 20 minutes in PBST, then incubated for 10 minutes in PBST with rhodamine-conjugated phalloidin (1:1000, Molecular Probes) and DAPI (1:1000, Molecular Probes). After manual removal of stage-14 egg chambers, samples were mounted using the *Slowfade Light* Antifade Kit (Molecular Probes).

Results

Generation of germline mosaics

To determine whether expression of *grk* in the oocyte nucleus is required for axial patterning, we generated egg chambers with germlines mosaic for a null allele of *grk, grk^{2B6}*, using the FLP/FRT system to drive site-specific mitotic recombination in heterozygous females (Fig. 1A). Recombination events occurring during the division of heterozygous germline stem cells result in germline clones, in which the germline cyst consists entirely of homozygous cells, whereas recombination during subsequent germline divisions gives rise to mosaic cysts, with both wild-type and mutant cells. To address the requirement for *grk* transcription in the oocyte nucleus, we analyzed mosaics in which some cells were homozygous for the *grk^{2B6}* mutation and others retained *grk* function. If *grk* were produced exclusively by the oocyte nucleus, then egg chambers lacking a functional copy of the *grk* gene in the oocyte would be predicted to exhibit the AP and DV patterning defects characteristic of *grk* homozygotes, even in the presence of wild-type nurse cells.

As a genetic marker for these mosaics, we used a transgene expressing a nuclear form of GFP. This marker was placed in *trans* to the *grk^{2B6}* allele, so that homozygous mutant cells were marked by the lack of GFP (Fig. 1B). Egg chambers with homozygous mutant germline clones were readily recognized by the absence of detectable GFP fluorescence throughout the germline, while mosaic cysts exhibited a combination of nuclei with and without GFP. Though well established as a marker for clonal analysis, we anticipated that the use of nuclear GFP as a genotypic marker in mosaic cysts would be less straightforward. Due to the transport of material from the nurse cells to the developing oocyte through the cytoplasmic bridges connecting the germline cells, any GFP in the oocyte nucleus could include a contribution from the nurse cells. Moreover, it was unclear whether the GFP transgene would be expressed in the transcriptionally inactive oocyte nucleus. To circumvent these issues, we avoided assessing the genotype of the oocyte directly by restricting our analysis to egg chambers in which half the cells were homozygous wild type, as determined by uniformly high levels of GFP, and half were homozygous mutant, as determined by the lack of GFP. Given that the germline mitoses occur in an invariant pattern, mitotic recombination in the first division will always produce precisely eight cells of each genotype. Therefore in egg chambers with eight homozygous wild-type nurse cells, with uniformly high nuclear GFP, and seven *grk^{2B6}* homozygous nurse cells, with little or no GFP, we could

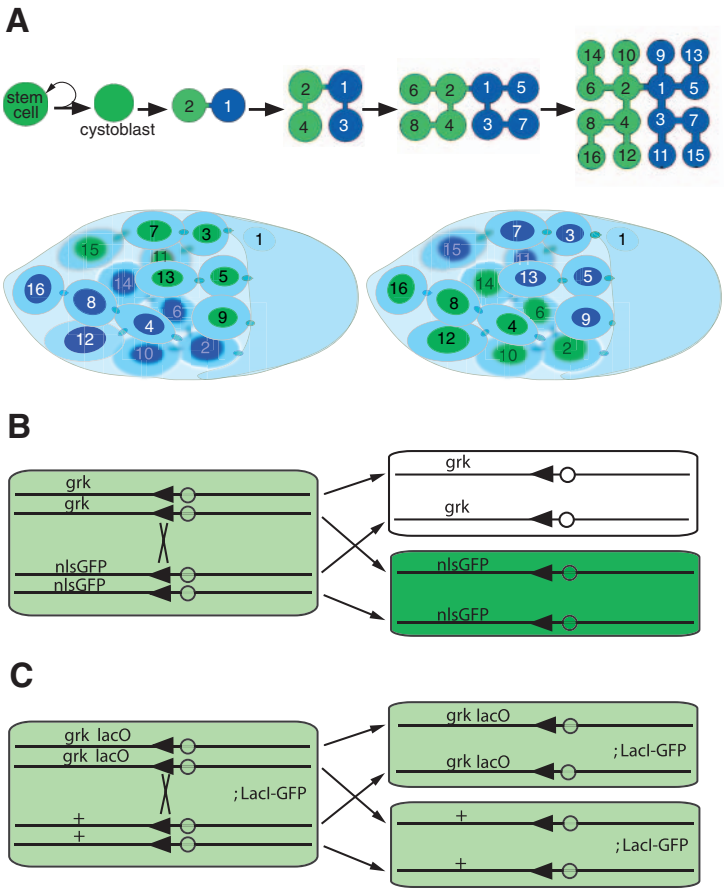

Fig. 1. Generation of germline mosaics. (A) Each germline cyst arises from a single cystoblast through four consecutive rounds of mitosis. Incomplete cytokinesis after each mitosis results in a stereotyped pattern of cytoplasmic bridges between the 16 cells of the cyst. Either of the two cells with four ring canals will become the oocyte. Recombination in the germline stem cell divisions generates clonal germline cysts (not shown), while mitotic recombination during the first division of a heterozygous cystoblast results in a mosaic cyst consisting of eight wild-type cells (green) and eight mutant cells (blue). In such mosaics, the oocyte nucleus can be either wild type (left) or mutant (right). (B) Generation of mosaics with the nuclear GFP marker. Induction of the Flp recombinase (not shown) in heterozygous cells (left, shown after DNA replication but before mitosis) mediates mitotic recombination at FRT sites (triangles), resulting in homozygous daughter cells. *grk^{2B6}/grk^{2B6}* cells are marked by the lack of GFP expression. (C) In the *lacO*/GFP-LacI system, the starting heterozygous cells contain the *lacO* transgene in *cis* to the *grk^{2B6}* mutation, as well as the GFP-LacI transgene. All cells exhibit nuclear GFP-LacI fluorescence, and discrete fluorescent foci are visible in the nuclei of cells with the lacO transgene. Heterozygous females exhibit a single focus of GFP in the oocyte nucleus and multiple foci in the polyploid nurse cells. Homozygous wild-type daughter cells lack these foci, while *grk^{2B6}/grk^{2B6}* oocyte nuclei exhibit two foci.

infer clearly that the oocyte must be the eighth mutant germline cell. In the reciprocal mosaics, with uniformly high nuclear GFP in seven nurse cells and little or none in the remaining eight, we concluded that the oocyte must be the eighth homozygous wild-type cell.

The four consecutive mitoses that generate each germline cyst result in a configuration of germline cells in which the oocyte is directly connected to four of the nurse cells: one is its sister from the first mitosis and the other three are its daughters from the subsequent rounds (see Fig. 1A). In 130/131 examples of germline mosaics with half wild-type and half mutant cells, we noted that three of these four nurse cells shared the deduced oocyte genotype, while the fourth had the opposite genotype. This nearly invariant configuration confirms our assessment of oocyte genotypes and demonstrates that these mosaics are generated primarily by recombination events occurring during the first round of germline mitoses. Multiple recombination events in subsequent mitoses probably generated the single exceptional mosaic.

In parallel, to determine the genotype of the oocyte directly, we generated germline mosaics using a transgene containing 256 direct repeats of the *lac* operator (*lacO*) as a genetic marker (Robinett et al., 1996; Straight et al., 1996; Vazquez et al., 2001). The presence of the transgene was visualized by the binding of a nuclear GFP-tagged Lac repressor protein (GFP-LacI), which binds to the *lacO* transgene and yields a discrete focus of nuclear fluorescence. In this system, the integrated *lacO* transgene itself functions as the genotypic marker, and is therefore strictly cell-autonomous. In ovaries from females heterozygous for the *lacO* transgene, a single focus was present in the oocyte nucleus, whereas in the nurse cell nuclei, which are highly polyploid with partially dispersed chromatids, multiple foci were visible. For generation of germline mosaics, we constructed a chromosome containing the *lacO* transgene in *cis* to the grk^{2B6} allele, so that homozygous *grk* mutant cells would be homozygous for the transgene as well. This configuration allowed us to recognize a homozygous mutant oocyte directly by the presence of two clear foci of GFP fluorescence within the oocyte nucleus (Fig. 1C).

Synthesis of *grk* in the oocyte nucleus is not required for AP axis establishment

We examined whether production of *grk* in the oocyte nucleus is required for AP patterning of the egg chamber by observing the position of the oocyte nucleus. As described above, the movement of the oocyte nucleus from a central position at the oocyte posterior to an asymmetric location at the anterior cortex requires the correct polarization of the oocyte microtubule network, which in turn depends upon induction of posterior follicle cell fates by Grk signaling in early oogenesis. In the absence of *grk* function, this sequence of events is not initiated and the oocyte nucleus remains at the posterior of the oocyte (Gonzalez-Reyes et al., 1995; Roth et al., 1995).

In egg chambers with germline clones, in which all germline cells were homozygous for the grk^{2B6} allele and therefore lacked *grk* function, the oocyte nucleus was located at the posterior of the oocyte in 94/154 cases observed (61%; Fig. 2A). This frequency of mislocalization is consistent with previous analyses, which report posterior localization of the oocyte nucleus in 31-70% of egg chambers from homozygous

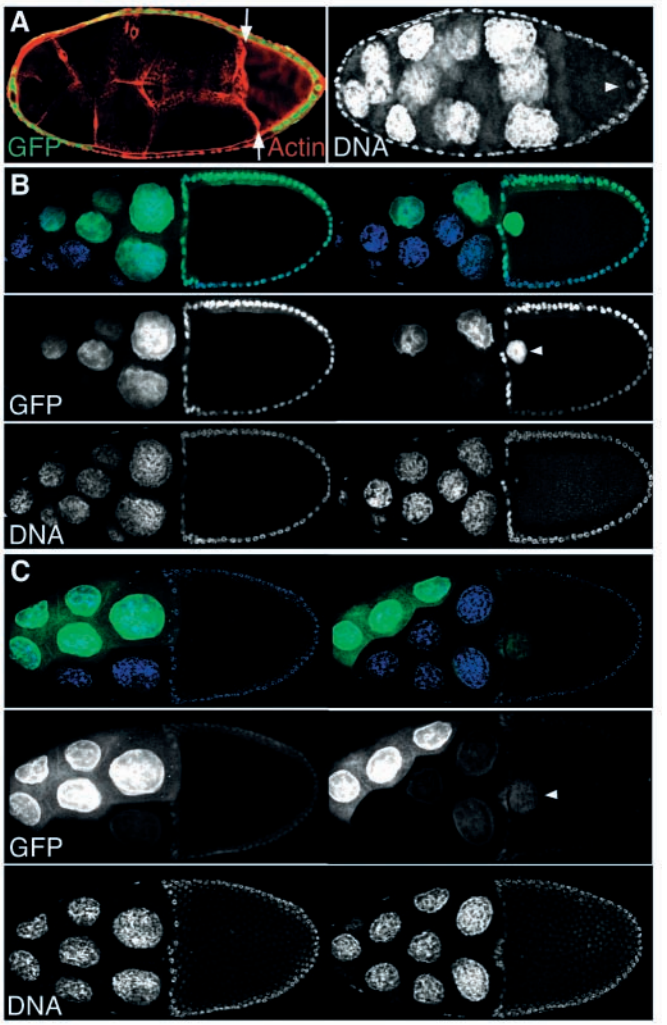

Fig. 2. The presence of a wild-type *grk* allele in the oocyte is not required for AP axial polarity. (A) grk^{2B6}/grk^{2B6} germline clone. The oocyte nucleus (arrowhead) has not migrated to the anterior cortex (arrows), reflecting a defect in Grk-mediated AP polarity determination. (B) Two focal planes of a dorsal view of a single egg chamber with a mosaic germline composed of eight grk^{2B6}/grk^{2B6} nurse cells (dark) and seven homozygous wild-type nurse cells (bright green); the oocyte is homozygous wild type. The oocyte nucleus (arrowhead) is properly localized at the anterior. (C) Two focal planes of a dorsal view of a single egg chamber with a mosaic germline composed of eight homozygous wild-type nurse cells (bright green) and seven grk^{2B6}/grk^{2B6} nurse cells (dark); the oocyte is grk^{2B6}/grk^{2B6}. Although the oocyte cannot itself produce *grk*, the posterior to anterior migration of the oocyte nucleus is normal (arrowhead).

grk mutant females, depending on the allelic combination (Gonzalez-Reyes et al., 1995; Roth et al., 1995). This defect confirms the effect of the grk^{2B6} mutation on *grk* function and provides an important internal control for the analysis of egg chambers with mosaic germlines, which are recovered from the same females. We then analyzed GFP-marked germline mosaics consisting of eight homozygous wild-type and eight homozygous mutant cells. In 21/21 control mosaics, in which the oocyte was homozygous for the wild-type *grk* allele, the oocyte nucleus was properly localized at the anterior margin

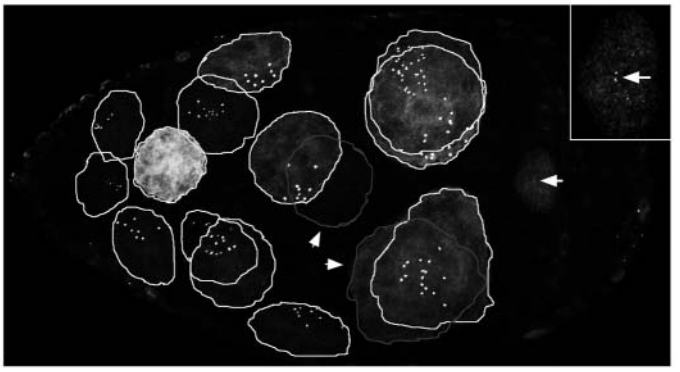

Fig. 3. Mosaics marked with the *lacO*/GFP-LacI system confirm that *grk* production in the oocyte is not required for oocyte AP polarity. Two foci of nuclear GFP-LacI fluorescence are visible in the oocyte nucleus (arrow), indicating that it is homozygous for the grk^{2B6} allele. Multiple foci are visible in 13 nurse cell nuclei and two nuclei exhibit no foci (arrowheads). Note that GFP-LacI levels typically vary among nuclei. Though one nucleus has high GFP-LacI and some nuclei overlap in this projection of confocal optical sections, the presence or absence of foci in each nucleus was determined by inspection of individual sections at the appropriate exposure. Inset shows an enlargement of the oocyte nucleus.

of the oocyte (Fig. 2B). Strikingly, anterior localization of the oocyte nucleus was also observed in 22/22 mosaics in which the oocyte was homozygous for the grk^{2B6} allele (Fig. 2C). These observations indicate that transcription of *grk* in the oocyte nucleus is not required for oocyte AP polarity.

We confirmed these observations using the *lacO*/GFP-LacI system as a genotypic marker. Fig. 3 shows a mosaic egg chamber in which the oocyte exhibited two foci of GFP-LacI fluorescence, indicating that it was homozygous for the grk^{2B6} allele (see Fig. 1C). In addition, 13 nurse cells exhibited multiple foci and two nurse cells lacked foci. We can conclude from the lack of foci in two nurse cell nuclei that these cells were homozygous for the wild-type *grk* allele, and that this mosaic configuration arose from a single recombination event during the third cystoblast division or two events during the final division. Although the oocyte lacked any functional copies of the *grk* gene, the anterior migration of the oocyte nucleus was not affected. Proper localization of the oocyte nucleus was observed in 4/4 germline mosaic egg chambers with a *grk* mutant oocyte detected using this system, corroborating our results with the nuclear GFP marker.

Taken together, our analysis of germline mosaics indicates that synthesis of *grk* in the oocyte is not required for its function in determination of the polarity of the oocyte AP axis. We therefore conclude that in wild-type egg chambers *grk* transcription is not restricted to the oocyte nucleus and that production of *grk* in the nurse cells is sufficient for proper *grk* function in this process.

Synthesis of *grk* in the oocyte nucleus is not required to establish the oocyte DV axis

Coincident with the movement of the oocyte nucleus to the anterior cortex of the oocyte, *grk* mRNA and protein also relocalize, remaining closely associated with the oocyte nucleus and achieving an asymmetric anterior localization that

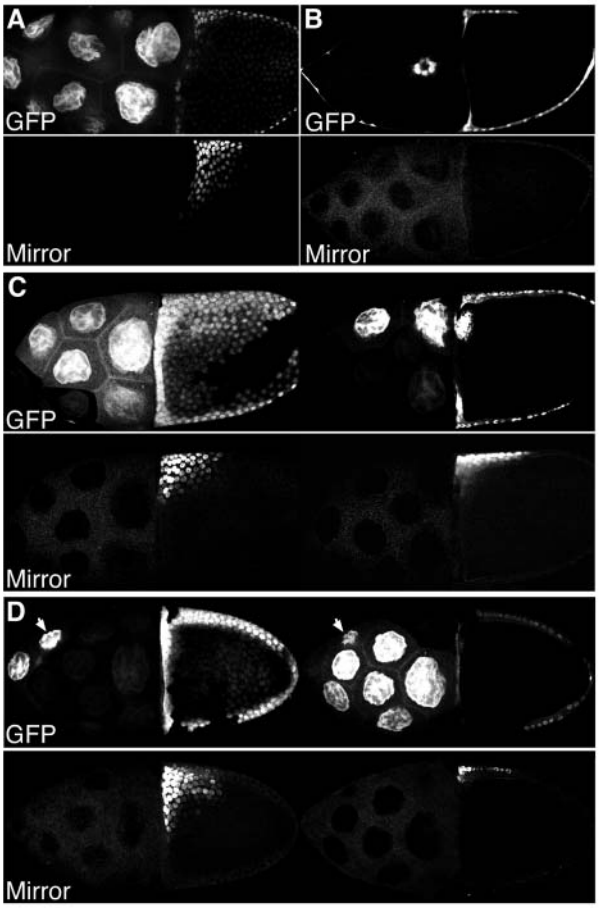

Fig. 4. Production of *grk* in nurse cells is sufficient to establish the oocyte DV axis. (A) In wild-type egg chambers, *mirr-lacZ* is expressed in dorsal anterior follicle cells in response to Grk-Egfr signaling. (B) *mirr-lacZ* is not expressed in egg chambers with grk^{2B6}/grk^{2B6} germline clones, confirming that this cell fate marker is *grk*-dependent. (Note that the group of small GFP-positive cells is the migrating border cell cluster.) (C) Two focal planes of a germline mosaic egg chamber with a homozygous wild-type oocyte. *mirr-lacZ* is expressed in dorsal anterior follicle cells. (D) Two focal planes of a germline mosaic egg chamber with a grk^{2B6}/grk^{2B6} oocyte; arrows indicate a single nucleus that is visible in both images. This egg chamber is slightly rotated laterally with respect to that shown in C, accounting for the minor apparent difference in expression pattern. There was no significant difference in the mean (±s.d.) number of *mirr-lacZ*-positive nuclei between germline mosaics with a mutant oocyte (165±46) and either mosaics with wild-type oocyte (205±43) or *grk* heterozygotes (158±55).

defines the dorsal side of the oocyte and thus the DV axis of the egg chamber. While the data presented above indicate that the production of *grk* by the nurse cells is sufficient for AP patterning, this analysis does not address the possibility that, at later stages of oogenesis, transcription of *grk* in the oocyte nucleus is required for DV patterning. Indeed, it has been speculated that *grk* may be produced in all germline cells during early stages, for *grk*-mediated AP patterning, then produced primarily or exclusively in the oocyte nucleus in later stages, leading to the dorsally restricted distribution required for DV patterning (MacDougall et al., 2003; Norvell et al., 1999; Thio et al., 2000).

To investigate this possibility, we assessed dorsal follicle cell fate determination in *grk* mosaics. As a cell fate marker we used an enhancer trap inserted in the *mirror* locus, *mirr-lacZ*, which drives expression of a *lacZ* reporter gene in dorsal anterior follicle cells in response to Grk-Egfr signaling (Fig. 4A) (Jordan et al., 2000; Zhao et al., 2000). Egg chambers with germline clones homozygous for the grk^{2B6} mutation exhibited no follicle cell expression of *mirr-lacZ*, confirming that expression of this reporter is *grk*-dependent (Fig. 4B). By contrast, in germline mosaic egg chambers derived from the same females, expression of the *mirr-lacZ* marker was detected in dorsal anterior follicle cells, even when the oocyte contained no functional copies of the *grk* gene (Fig. 4C,D). We found no significant difference in the number of *lacZ*-positive follicle cells between stage-10 germline mosaics with a *grk* mutant oocyte (n=12) and germline mosaics with either a wild-type oocyte (n=7) or grk^{2B6} heterozygous egg chambers (n=25), from which germline mosaics are derived. While these data reveal no gross alterations in DV patterning in the absence of a functional *grk* gene in the oocyte nucleus, because the *mirr-lacZ* expression pattern is dynamic and the number of positive cells varies even among stage-10 egg chambers from *grk* heterozygotes, any subtle changes in expression within this range would be difficult to detect.

As an additional marker of DV patterning, we analyzed the expression pattern of the Broad Complex (BR-C) protein. Although BR-C is initially expressed throughout the follicular epithelium, this expression pattern is refined during stage 10, in response to Grk-Egfr signaling, resulting at the end of this stage in two dorsolateral groups of follicle cells with high levels of BR-C flanking a dorsal midline region where BR-C levels are eliminated (Deng and Bownes, 1997; Suzanne et al., 2001; Tzolovsky et al., 1999). This pattern of BR-C expression was also observed in germline mosaic egg chambers with a homozygous grk^{2B6} oocyte (Fig. 5), further indicating that synthesis of *grk* in the oocyte nucleus is not required to determine the DV axis of the egg chamber. To examine the BR-C pattern more closely, we counted the number of cells along the AP and DV dimensions of the dorsal midline domain. On average, this domain was approximately one cell shorter along both axes in germline mosaics with a mutant oocyte than in those with a wild-type oocyte (see Fig. 5). However, the ranges of these dimensions overlapped, and the dimensions of this domain for all mosaics fell within the range observed in non-mosaic heterozygous egg chambers at the same stage. Therefore, while the difference in BR-C expression between mosaics with a wild-type and mutant oocyte could represent a minor or late contribution of *grk* by the oocyte nucleus, the variability at this stage probably reflects the dynamic nature of the BR-C expression pattern and limits by definition the resolution of this analysis, precluding a definitive conclusion regarding possible subtle patterning differences.

Taken together, these results demonstrate that the nurse cells contribute sufficient *grk* to establish the DV axis of the egg chamber. While our data do not exclude the possibility that the oocyte also produces *grk*, these findings clearly indicate that DV patterning is largely normal even in the absence of a functional *grk* allele in the oocyte nucleus.

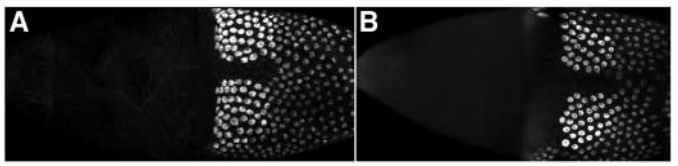

Fig. 5. Production of *grk* in the oocyte is not required for induction of dorsal follicle cell fates. Dorsal view of the BR-C expression pattern in germline mosaics with (A) a wild-type oocyte (confocal image) or (B) a grk^{2B6}/grk^{2B6} oocyte. Measurement of the dorsal midline domain that lacks BR-C expression revealed that the mean (±s.d.) dimensions of this domain were 11.3 (±1.3) cells along the AP axis and 3.4 (±0.7) cells along the DV axis (n=15) in mosaics with a wild-type oocyte, 10.2 (±1.0) cells along the AP axis and 2.3 (±0.6) cells along the DV axis (n=12) in mosaics with a *grk* mutant oocyte, and 10.9 (±1.6) cells along the AP axis and 3.3 (±0.7) cells along the DV axis in *grk* heterozygotes (not shown). Although this domain is approximately one cell shorter along both axes in mosaics with a *grk* mutant oocyte, the resolution of this assay is limited by the dynamic nature of the BR-C expression pattern, as reflected in the range observed in *grk* heterozygotes.

Border cell migration does not require synthesis of *grk* in the oocyte

Grk-Egfr signaling is also required to guide the migration of a specialized subpopulation of follicle cells called border cells. This cluster of cells delaminates from the anterior follicular epithelium at stage 9 of oogenesis and migrates posteriorly, between the nurse cells, toward the oocyte. Upon reaching the anterior margin of the oocyte, the border cells migrate dorsally and assume a position adjacent to the oocyte nucleus (Spradling, 1993). Loss of Grk function in the germline or Egfr function in the border cells themselves leads to the failure of the dorsal phase of this migration, indicating that Grk provides a spatial cue that acts through Egfr to guide border cell migration (Duchek and Rorth, 2001).

To determine whether synthesis of *grk* in the oocyte nucleus is required for border cell guidance, we examined the position of the border cell cluster in germline mosaic egg chambers of the appropriate stage. Border cells were dorsally localized along the anterior margin of the oocyte in 12/14 germline mosaics with a wild-type oocyte, comparable to previous observations of wild-type egg chambers (Duchek and Rorth, 2001). In germline mosaics with a *grk* mutant oocyte, the border cell cluster was dorsally localized in 14/15 cases. These data indicate that synthesis of functional *grk* transcripts in the oocyte nucleus is not required to guide border cell migration.

Production of *grk* in the oocyte nucleus is not required for localization of Grk protein

The observation that AP and DV patterning occur normally in germline mosaics with a homozygous *grk* mutant oocyte suggests that the Grk signal is properly localized in these egg chambers. To test this prediction, we visualized the localization of the Grk protein in mosaic egg chambers using a monoclonal antibody. No specific immunoreactivity was observed in 14/14 homozygous mutant germline clones, confirming the specificity of the antibody (data not shown). In germline mosaics, the localization of Grk in those with a homozygous mutant oocyte (Fig. 6B,C) was indistinguishable from that of those with a wild-type oocyte (Fig. 6A). This observation indicates that normal Grk localization is achieved even when the transcript is derived exclusively from the nurse cells and indicates that

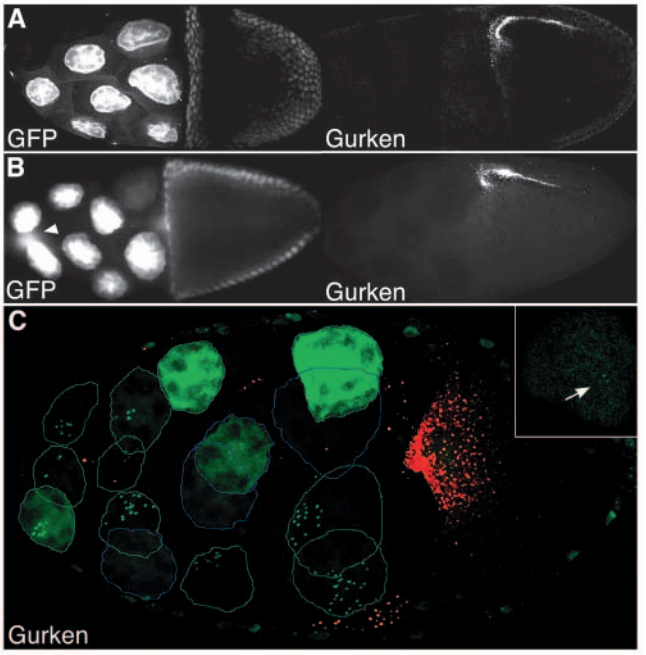

Fig. 6. Nurse-cell-derived *grk* transcripts generate properly localized Grk. (A,B) Grk protein is properly localized in nuclear GFP-marked germline mosaics with either a wild-type (A, confocal image) or grk^{2B6}/grk^{2B6} (B) oocyte. The arrow in B indicates a nurse cell nucleus with high levels of GFP that lies behind the focal plane shown. (C) Dorsal view of a germline mosaic with the lacO/GFP-LacI genotypic marker. The oocyte nucleus exhibits two foci of GFP fluorescence (inset), while four nurse cell nuclei lack foci (outlined in blue; the presence or absence of foci was determined by inspection of individual confocal sections at the appropriate exposures). Although this oocyte is homozygous for the grk^{2B6} allele, Grk (red) exhibits a normal dorsal anterior localization typical of this stage.

production of wild-type *grk* transcripts in the oocyte is not required to generate a spatially restricted Grk signal.

Discussion

Transcription of *grk* in the nurse cells is sufficient for AP and DV axis formation

Our analysis of genetic mosaics demonstrates that the presence of a functional copy of the *grk* gene in the oocyte nucleus is not required for *grk* function, indicating that the nurse cells provide *grk* to the oocyte. Germline mosaics are generated through mitotic recombination in heterozygous cells during the formation of the 16-cell germline cyst; each homozygous mutant cell is therefore ultimately derived from a heterozygous cystoblast. However, it is unlikely that sufficient *grk* from the cystoblast would persist through these mitoses, and the substantial subsequent increase in cell size, to account for the normal patterning observed in germline mosaics with a *grk* mutant oocyte. First, no *grk* mRNA or protein is present during the germline mitoses that generate each cyst, becoming detectable only later, as the complete 16-cell cyst becomes enveloped by the follicular epithelium (Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1996). Moreover, any sub-detectable levels of *grk* present earlier would not be predicted to be sufficient for patterning, as *grk* is weakly haploinsufficient: even *grk* heterozygotes, where *grk* is readily detectable, display some egg chamber patterning defects (Neuman-Silberberg and Schüpbach, 1994). Therefore our data indicate that transcription of the *grk* gene occurs in the nurse cells and that this contribution can generate a properly localized Grk signal that is sufficient to mediate axial patterning of the oocyte.

Does the oocyte nucleus produce *grk*?

Evidence for transcription of *grk* in the oocyte nucleus has been reported previously (Saunders and Cohen, 1999). After treatment of females with colchicine to disrupt microtubule-based transport from the nurse cells to the oocyte, *osk* transcripts were retained in the nurse cells while *grk* transcripts were detected exclusively in the oocyte, consistent with *grk* transcription in the oocyte nucleus. However, it is also possible that there is a fundamental difference between *grk* and *osk* in the timing or mode of transport. For example, blocking transport of *grk* to the oocyte could require a level of microtubule disruption equivalent to that which would disrupt oocyte specification (Koch and Spitzer, 1983), potentially confounding analysis by this method. Alternatively, transport may occur via a microtubule-independent mechanism, or along microtubules that are stable and therefore insensitive to disruption by treatment with colchicine, which affects only dynamic microtubules. Such a population of stable microtubules, present at least in the early stages of oogenesis, has recently been described (Roper and Brown, 2004).

In a complementary approach, transcripts from a reporter construct under the control of the *grk* promoter were observed to accumulate exclusively in the oocyte, consistent with activity of this promoter exclusively in the oocyte nucleus (Saunders and Cohen, 1999). However, subsequent mapping of *grk* transcripts suggested that the *grk* transcription start site is located farther upstream than previously recognized (Thio et al., 2000), indicating this construct probably contained elements present within the *grk* 5′ UTR. While there is no evidence that this sequence is relevant to mRNA transport or localization, strong conclusions about *grk* promoter activity cannot be drawn.

Our genetic approach demonstrates that the nurse cells provide *grk* to the oocyte. However, because it is difficult to detect subtle or late changes in patterning, our data do not exclude a contribution of *grk* by the oocyte nucleus. Given the lack of obvious defects in mosaics with a *grk* mutant oocyte, and the greater synthetic capacity of the polyploid nurse cells, it seems likely that any *grk* contribution by the oocyte nucleus would be minor. Nevertheless, the important conclusion of this work is the clear demonstration that the nurse cells transcribe *grk,* and that these transcripts are sufficient for normal Grk localization and the establishment of the oocyte AP and DV axes. These observations imply the existence of a mechanism for their transport from the nurse cells and subsequent dorsal anterior localization within the oocyte.

Implications for *grk* mRNA localization

We have been unable to visualize the distribution of *grk* mRNA in germline mosaics, due to the incompatibility of GFP fluorescence with conditions required for in-situ hybridization and the inconsistent levels of signal obtained with available anti-GFP antibodies. However, it seems likely that the normal

distribution of Grk in mosaics with a mutant oocyte reflects proper localization of *grk* mRNA. The alternative possibility, that *grk* mRNA is mislocalized in mosaics with a *grk* mutant oocyte but yields a properly localized protein, would imply that synthesis of wild-type *grk* transcripts in the oocyte nucleus is required to localize nurse-cell-derived transcripts. Although there is evidence for translational regulation of unlocalized *grk*, which would account for the normal Grk distribution observed in such mosaics (Norvell et al., 1999), it is unclear how localization of *grk* transcripts from the nurse cells would depend on additional *grk* production in the oocyte nucleus. Although we cannot exclude such a model, due to its complexity it seems less likely.

The site of *grk* transcription has important implications for the consideration of potential localization mechanisms. While the localization of the *grk* mRNA between the oocyte nucleus and adjacent cortex has led to the proposal that this distribution arises from *grk* transcription in the oocyte nucleus and local anchoring of *grk* transcripts (Goodrich et al., 2004; Norvell et al., 1999; Palacios and St Johnston, 2001; Saunders and Cohen, 1999), such a model would not address the proper localization and patterning function of *grk* contributed by the nurse cells. As *grk* encodes a secreted protein, it has also been suggested that a dorsal anterior concentration of exocytic pathway components within the oocyte could contribute to its localization. A careful analysis of transitional endoplasmic reticulum and Golgi compartments, however, reveals a uniform distribution and indicates that polarized Grk distribution is driven by the localization of its mRNA (Herpers and Rabouille, 2004).

The mechanism of transport of *grk* transcripts from the nurse cells to the oocyte is unknown. However, within the oocyte, proper dorsal anterior localization of *grk* mRNA requires the heterogeneous nuclear ribonuclear protein (hnRNP) proteins Sqd (also known as Hrp40) and Hrb27C (also known as Hrp48), which bind to the 3′ UTR of the *grk* mRNA (Goodrich et al., 2004; Neuman-Silberberg and Schüpbach, 1993; Norvell et al., 1999). These hnRNPs form a complex with the nascent *grk* transcript, then recruit cytoplasmic proteins to the *grk* RNP complex upon its export from the nucleus to regulate *grk* localization and translation in the cytoplasm (Goodrich et al., 2004; Norvell et al., 1999). Although this model proposes that the interaction of Sqd and Hrb27C with the *grk* transcript occurs in the oocyte nucleus (Goodrich et al., 2004; Norvell et al., 1999), it seems plausible to suggest that these complexes assemble in the nurse cells to regulate *grk* localization within the oocyte. Localization of *grk* mRNA within the oocyte also requires transport on microtubules, because disruption of the minus-end-directed microtubule motor cytoplasmic Dynein results in defects in *grk* localization (Brendza et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002; MacDougall et al., 2003). It is unclear, however, whether these factors are required for transport of *grk* into the oocyte, as the most obvious consequence of loss of their function is mislocalization of *grk* mRNA to the oocyte anterior margin.

Taken together with previous work, our data favor a model in which *grk* transcripts are assembled in the nurse cell nuclei into hnRNP particles containing Sqd and Hrb27C, followed by recruitment upon nuclear export of cytoplasmic factors regulating localization and translation. These *grk*-containing complexes would ultimately associate with microtubule motors, resulting in minus-end-directed transport along microtubules emanating from the dorsal anterior region; indeed there is evidence for a scaffold of microtubules around the oocyte nucleus (Clark et al., 1997; MacDougall et al., 2003; Theurkauf et al., 1992). Remaining to be resolved, however, is a mechanism that would distinguish microtubule-based localization of *grk* from that of anteriorly localized messages that are not dorsally restricted. Although *grk* could interact with specific *trans*-acting factors in a distinct hnRNP particle, a potential sorting mechanism would nevertheless require differences in the dorsally oriented microtubules to allow recognition by motors carrying *grk*-containing particles. While modifications of tubulin itself or the association with distinct microtubule-associating proteins could distinguish microtubule networks (Westermann and Weber, 2003), the mechanism underlying the sorting of *grk* from other anterior transcripts remains to be determined.

We thank Stefan Luschnig, Donald Morisato, Trudi Schüpbach and Julio Vazquez for fly strains, Greg Guild for the anti-BR-C antibody, Cheryl Van Buskirk and K. Nicole Clouse for advice on immunohistochemistry, and Jennifer Thompson for technical assistance. We are also grateful to Trudi Schüpbach, Paul Lasko and David Dansereau for helpful comments on the manuscript. The monoclonal antibodies 1D12 and 40-1a, developed by Trudi Schüpbach and Joshua Sanes, respectively, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. L.C. and L.N. were supported by the Canada Research Chairs Program (www.chairs.gc.ca).

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