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The role of maternal and zygotic *Gprk2* expression in *Drosophila* development

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Abstract

G protein-coupled receptor activity is controlled by a number of factors including phosphorylation by the family of G protein-coupled receptor kinases. This phosphorylation is an important first step in desensitization of the receptor. The role of G protein-coupled receptor kinases in cellular physiology has been extensively studied, but less is known about their role in development. A *Drosophila* G protein-coupled receptor kinase mutant (*gprk2*^{6936}) has developmental defects throughout the life cycle of the fly. This allows the opportunity to address G protein-coupled receptor kinase's function in vivo. Using a series of transgenic flies in which the wild type *Gprk2* gene is expressed under the control of the *hsp70* or germline-specific promoter, in combination with germline mosaic analysis, we have made a detailed analysis of the developmental stages in which *Gprk2* expression is required and the tissues that must express *Gprk2* for rescue of the *gprk2*^{6936} mutant. These studies have shown that *Gprk2* expression is required in the germline for proper formation of the anterior egg structures and for early embryogenesis. In the absence of maternal *Gprk2* activity, zygotic expression affords partial rescue of egg hatching, suggesting that *Gprk2* also plays an important role in late embryogenesis.

Keywords: G protein-coupled receptor kinase; GRK; GPRK2; *Drosophila*; Oogenesis; Embryogenesis; Development

Signaling through G protein-coupled (or heptahelical) receptors is used extensively in the development of eukaryotic animals. Members of the heptahelical superfamily respond to extracellular signals and initiate intracellular signaling through the activation of heterotrimeric G proteins. The desensitization of heptahelical receptors, a mechanism by which these receptors cease to respond to a continued stimulus, can be mediated by G protein-coupled receptor kinases (GRKs). GRKs specifically phosphorylate the activated, or agonist-occupied, forms of receptors. Phosphorylation results in uncoupling of the receptor and G protein, and may also control receptor internalization and down-regulation [1]. Because GRKs represent an important form of receptor regulation, it is not surprising that the activity of GRKs, themselves, is tightly regulated. The biochemical functions of the GRKs have been extensively studied in vitro. The activity of different GRKs can be controlled by subcellular localization, phosphorylation, and by interaction with proteins such as actin, calmodulin, and caveolin [2,3]. GRKs can also phosphorylate soluble substrates such as β-tubulin, suggesting that the function of these kinases is more complicated than what was previously understood [4].

More recently, the in vivo functions of GRKs have been addressed through genetic manipulations [5,6]. In *Drosophila melanogaster*, a mutation in the *G protein-coupled receptor kinase 2* (*Gprk2*) gene, which encodes a putative member of the GRK family, causes multiple defects in oogenesis and embryogenesis [7,8]. Because of the relative ease of genetic manipulation in *Drosophila*, this organism offers an excellent opportunity to characterize the requirement for GRKs in the developing animal.

The developmental defects in the *Gprk2* mutant (*gprk2*^{6936}) have been best characterized in the ovaries.

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The *gprk2*^{6936} mutation is caused by a P element insertion in the first exon of the gene. In the wild type fly, expression of GPRK2 protein is detectable in germ cells (particularly the oocyte) throughout oogenesis, but not in the somatic follicle cells. GPRK2 protein is also detectable in most non-ovarian tissues. In most tissues, GPRK2 protein expression is associated with the membrane. In the *gprk2*^{6936} mutant, expression in the ovaries and brain is no longer detectable. Homozygous *gprk2*^{6936} females produce a very small number of adult progeny, suggesting that the *Gprk2* gene is required during development. Egg chambers isolated from mutant *gprk2*^{6936} ovaries display multiple defects including misshapen dorsal appendages, incomplete cytoplasmic dumping, and degeneration of egg chambers during mid-oogenesis. In addition, embryos that are laid by homozygous mothers hatch at a reduced rate compared to wild type. The unhatched embryos display a range of abnormal phenotypes including twisted gastrulation and fusion of segments [7].

To begin an analysis of *Gprk2* function in different tissues, we have explored the requirement for *Gprk2* expression in oogenesis and embryogenesis. Using rescue constructs driven by *hsp70* and germline-specific promoters, we found that expression of *Gprk2* is necessary and sufficient to rescue the semi-lethality, and the egg laying and hatching defects of the *gprk2*^{6936} mutant. *Gprk2* plays a role in early and late embryogenesis as shown by the requirement for both maternal and zygotic contributions. Germline clonal analysis suggests that a lack of expression in the germline is responsible for the morphological defects of the egg chambers and, in part, for the low level of hatching. These results highlight the importance of *Gprk2* in development and the need to better understand its regulatory role.

Materials and methods

Drosophila melanogaster stocks and transgenic flies. Flies were maintained on yeasted cornmeal/molasses media under standard culture conditions. The *gprk2*^{6936} mutant was isolated from a P element mutagenesis screen [9]. *Gprk2* transgenes were introduced into $w*;;e*$ homozygous flies by P element-mediated germ line transformation [10]. For each construct, at least two independent transformant strains were generated and tested. $w^*;;ry^{506}$ flies were used as the positive control for all experiments; w* is the background strain for the P element injections and ry^{506} is the background strain for *gprk2*^{6936}. Germline clones were generated using the technique of Chou and Perrimon [11]. To ensure that the effect seen with the rescue transgenes was not merely the result of a change in genetic background, all injections were made into the same strain, second chromosome insertions were used in every case, and statistical significance was determined in every experiment. Statistically significant differences between means were determined using t test and two tailed probabilities. A value of $P < 0.05$ was considered significant.

Plasmid construction. The heat-shock *Gprk2* construct ($P\{w^+$ *Gprk2*^{hs} = *hsp70-Gprk2*$\}$) was generated by inserting the *Gprk2* ORF (1135–3279 bp of the *Gprk2* cDNA; [6]) into the pCaSpeR-hs/act vector [12]. The *Gprk2* ORF was generated by PCR using *Pfu*I DNA polymerase (Stratagene) with forward primer (TGC GAC TAT TTG AAT TCG GGA GCG) and the reverse primer (CAT CCT CGA CTC TA GAT CTC TGC). Germline-specific expression constructs were generated by putting the *Gprk2* gene under the control of the *ovarian tumor* (*otu*) promoter in the pCOG vector [13]. The $P\{w^{+mC}$*Gprk2*^{otu} = *otu-Gprk2*$\}$ construct was made by subcloning a *Eco*RI/*Not*I fragment from the *hsp70-Gprk2* construct into the pCOG vector. The $P\{w^{+mC}$*Gprk2-HA*^{otu} = *otu-Gprk2-HA*$\}$ construct, which included a triple influenza hemagglutinin (HA) epitope ([MYPYDVPDYAGS] × 3) at the carboxyl-terminus, was also generated by PCR.The forward primer (TTC CGA GCA GGG AAT TCG CGG) and the reverse primer (TGA TCT CTG TCT AGA GCT TTC GAC CGT CGT GGA GGA C) generated a fragment containing 30–3266 bp of the *Gprk2* cDNA. This fragment was linked to a triple HA epitope with a stop codon, which was amplified from the vector pKH3 [14] (forward primer, ATT TAG GTG ACA CTA TAG and reverse primer, AAA CAA GTGCGG CCGCGG CGG TCA TCA ATT GAA TTC GGA TC). These two PCR-generated fragments were subcloned into the pCOG vector. All of the constructs were sequenced to determine the fidelity of the amplification and to ensure that all of the fragments were in-frame.

Testing of viability, egg laying, and hatching. To test for viability of rescued flies, $w;;$*gprk2*^{6936}/*TM3*, *Sb ry e* virgin females were mated to $w;;$*gprk2*^{6936}/*TM3*, *Sb ry e* males that carried one copy of the rescue P element, and the number of progeny were counted for 10–14 days and analyzed according to their genotypes. Although homozygous *gprk2*^{6936} adults emerge more slowly, in nearly every case, all adults emerged within a 14 day window. At least 125 progeny were counted in every case. Viability is given as a percentage of the expected number of progeny (i.e., with full rescue we would expect one-third of the progeny to be homozygous for *gprk2*^{6936}, therefore one-third is defined as 100%).

To test egg laying, virgin females that carried one copy of the rescue P element in a homozygous *gprk2*^{6936} background were first mated to $w;;ry^{506}$ or *gprk2*^{6936} males for 4–6 days at 24 °C. Individual females were transferred into new vials with 3–4 males and transferred every 24 h for 5 days. The number of eggs that were laid in each vial was counted.

To measure egg hatching, about ten 4–6-day-old females were placed in a vial together with 15–20 males of the desired genotype. After 24 h, egg collections were begun; five 24 h egg collections were obtained for each mating. From each collection, 100 eggs were laid out and the number of unhatched eggs was determined after 48 h at 24 °C.

Western blot analysis. Tissues were dissected in EBR (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 10 mM Hepes, pH 6.9) and were homogenized in ice-cold hypotonic buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA with 20 μg/ml phenylmethylsulfonyl fluoride). The samples were analyzed by Western blotting protocol as described previously [7]. Anti-HA antibodies (HA1.1 (16B12); Berkeley Antibody Co.) were used at a 1:800 dilution with 0.02% NaN_3 and incubated at room temperature for 1 h or overnight at 4 °C. The secondary antibody (goat anti-mouse-horseradish peroxidase, Amersham) was added at a dilution of 1:5000 in blocking buffer. The chemiluminescent detection of immune complexes on the blot was performed according to manufacturer's instructions (New England Nuclear).

Microscopy. Ovaries were dissected, fixed, and stained as described previously [7]. The HA1.1 monoclonal antibody was used at a dilution of 1:200 in PBS, 0.1% Triton X-100, 3% normal goat serum, and 0.05% NaN_3. After an overnight incubation at 4 °C, the samples were washed and incubated with Cy2-conjugated goat-anti-mouse antibodies (Vector Laboratories) at a 1:500 dilution. The specimens were cleared in 50% glycerol and mounted in Vectashield (Vector Laboratories). Images were collected on a Zeiss Axioskop and a Bio-Rad MRC-1024ES confocal microscope. All images were processed in Adobe Photoshop.

Results

Ectopic expression of the Gprk2 gene rescues the semi-lethality of the gprk2^{6936} mutant

The *gprk2*^{6936} mutant was identified based on its weak fertility. However, *gprk2*^{6936} is also semi-lethal (see Materials and methods); heterozygous *gprk2*^{6936} parents produce only 21–34% of the expected number of adult progeny (Fig. 1A). Although there are no deficiencies that uncover the *Gprk2* gene, molecular studies have suggested that *gprk2*^{6936} is not a null allele. In the *gprk2*^{6936} mutant, there is no detectable expression of GPRK2 protein in the brain or ovaries of the adult. However, there is expression in *gprk2*^{6936} adult carcasses (the entire fly minus the central nervous system and ovaries) by immunoblotting [7]. In addition, reverse transcription-PCR (RT-PCR) assays have confirmed that there is a low level of *Gprk2* expression in ovaries and non-ovarian tissues in the *gprk2*^{6936} mutant (S. Fan and L.E. Schneider, unpublished results). To determine if the decrease in *Gprk2* expression is responsible for the semi-lethality of the *gprk2*^{6936} mutant, we assayed viability in flies that express the *Gprk2* gene under the control of two different promoters. The *hsp70-Gprk2* construct was driven by the *hsp70* promoter and would be expected to yield ubiquitous expression. The *otu-Gprk2* and *otu-Gprk2-HA* constructs were driven by the *otu* promoter which drives expression in the germline but not in the follicle cells [13]. Because we did not know if blocking the end of the protein with an HA tag (*otu-Gprk2-HA*) would disrupt protein function, we generated a second *otu* construct with no tag (*otu-Gprk2*). Multiple lines were generated with each construct, and for each, two representative insertions on the second chromosome were selected for detailed study.

In measuring adult viability, we wanted to look only at zygotic function of the rescue construct. Therefore we crossed heterozygous *gprk2*^{6936} females with no rescue element to heterozygous *gprk2*^{6936} males carrying one copy of the rescue transgene. We then compared the number of homozygous *gprk2*^{6936} progeny with and without a copy of the transgene. All of the constructs rescued viability to a significant extent. The number of homozygous progeny with a copy of the transgene ranged from 63% to 100% of the expected number of progeny (Fig. 1A). Similar mating using males that were from the same *w*∗;;*e*∗ strain but carrying no insertion failed to rescue, demonstrating that rescue was not due to background mutations on the second chromosome.

The *otu-Gprk2* and *otu-Gprk2-HA* constructs rescued adult viability when inherited through the father. Because germline-specific products that are contributed by the father should not influence survival of the progeny, this rescue was most likely due to expression outside of the ovaries. The *otu* promoter has been shown to drive expression outside of the ovaries [15]. Similarly, immunoblotting studies demonstrated that *otu*-driven GPRK2-HA protein is expressed in adult heads and thoraces (data not shown). To confirm that the expression in the ovaries is restricted to the germline, we used the anti-HA antibody to examine expression in whole-mount tissues. As shown in Fig. 2, the GPRK2-HA

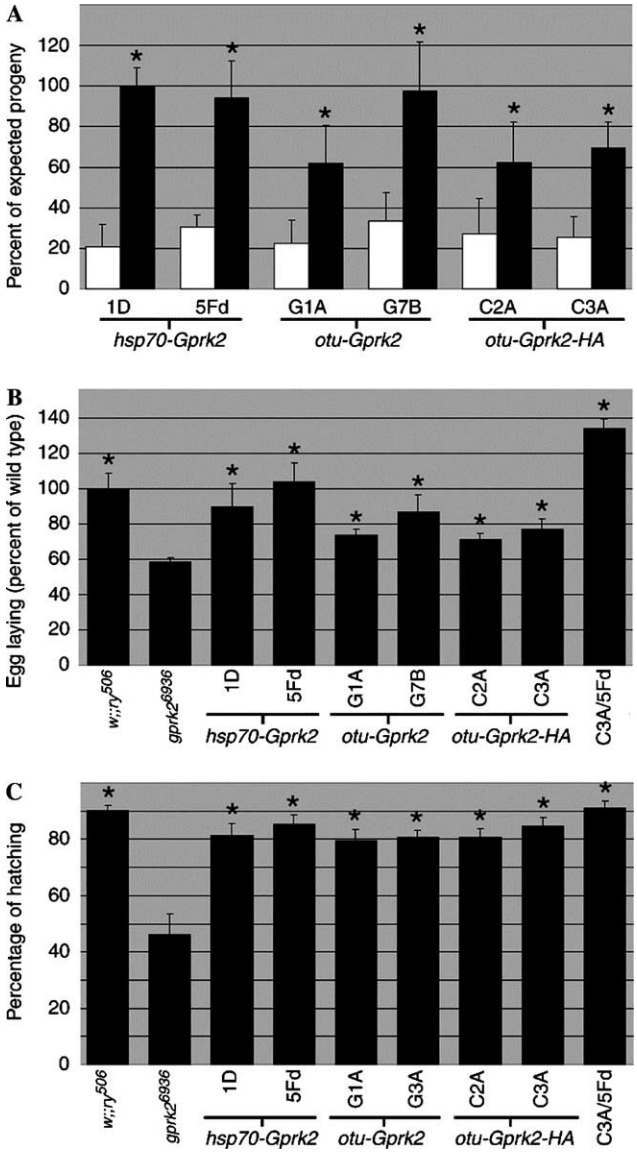

Fig. 1. Rescue of the *gprk2*^{6936} phenotype by wild type *Gprk2* constructs. (A) Rescue of viability. The bars represent the percentage of the expected number of adult progeny from a cross between heterozygous *gprk2*^{6936} parents. In each case, the percentage of *gprk2*^{6936} homozygous *gprk2*^{6936} progeny with (black bars) and without (white bars) one copy of a rescue transgene is compared. Asterisks represent a significant difference between each pair of bars ($P < 0.05$). (B,C) Rescue of egg laying and hatching. Homozygous females that were wild type (*w*;;*ry*^{506}) or homozygous for *gprk2*^{6936} with or without a single copy of a rescue construct (or one copy of two different constructs) were mated to wild type males and the number of eggs laid (B) and the hatching rate (C) was determined. The asterisks represent significant differences from *gprk2*^{6936} homozygotes.

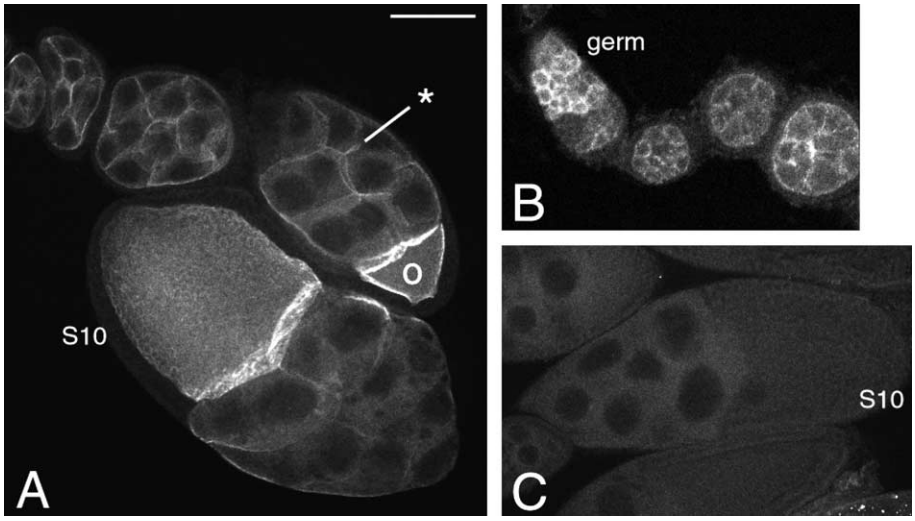

Fig. 2. Germline-specific expression of the *otu-Gprk2-HA* construct. Each image represents a stack of three adjacent, 10 μm confocal sections. Anterior is to the left in each case, except for the stage 10 (*S10*) egg chamber in (A). (A,B) Staining with an anti-HA antibody in ovaries of *otu-Gprk2-HA* lines. Expression begins in region 2A of the germarium (*germ*, in (B)) and continues throughout oogenesis. Immunoreactivity is associated with the membrane of the nurse cells (*asterisk*) and oocyte (*O*). In stage 10 (*S10*), expression decreases in nurse cells with a concomitant increase in staining in the oocyte that is frequently localized to the anterior end. This pattern of staining closely resembles the pattern of the endogenous protein (not shown). (C) No specific staining was observed in $w*;;ry^{506}$ flies. The scale bar is 100 μm for panels (A) and (C) and 50 μm for panel (B).

protein is expressed in the germline throughout oogenesis beginning at about stage 2A of the germarium. In early egg chambers, expression is highest at the nurse cell membranes. Beginning in stage 9, we see increasing expression in the oocyte, particularly at the anterior end. This pattern of expression closely mimics the endogenous GPRK2 protein [7], suggesting that the use of an exogenous promoter did not affect cellular distribution of the protein. The staining in follicle cells was indistinguishable from the negative control confirming that, within the ovaries, the *otu* promoter drives expression only in the germ cells.

In general, the rescue of viability was greater with the *hsp70-Gprk2* constructs than with the *otu-Gprk2* constructs, even when experiments were carried out in the absence of heat induction. To determine if this result was due to constitutive expression of the transgene, we used RT-PCR and primers specific for *hsp70-Gprk2*. These studies clearly showed that flies expressed the *hsp70-Gprk2* transgene in flies maintained at 18 °C (data not shown).

The hsp70-Gprk2 and otu-Gprk2 constructs rescue the fertility of the $gprk2^{6936}$ mutant

The rates of egg laying and hatching are both reduced in the $gprk2^{6936}$ mutant, leading to reduced fertility. Previous studies demonstrated that there was no expression of *Gprk2* mRNA and protein in the follicle cells, suggesting that follicle cell expression was not necessary for *Gprk2* function [7]. To test whether germline expression of *Gprk2* is necessary and sufficient for increased fertility, we carried out two sets of experiments. First, we compared the ability of the *hsp70-Gprk2* and *otu-Gprk2* constructs to rescue fertility of the $gprk2^{6936}$ mutant, using assays for both egg laying and hatching. Second, we generated germline clones of $gprk2^{6936}$ and tested fertility in those females.

To test egg laying, we crossed individual homozygous $gprk2^{6936}$ females that carried one copy of a rescue transgene to wild type males and determined the number of eggs laid as a percent of wild type. Homozygous $gprk2^{6936}$ females lay 58% as many eggs as wild type females (Fig. 1B). The presence of one copy of any of the constructs rescued egg laying. The different lines rescued to a varying degree, but in every case egg laying was significantly greater than in $gprk2^{6936}$ homozygotes. The rescue of egg laying was generally greater with the *hsp70-Gprk2* construct than with the *otu*-driven constructs. In addition, rescue by the two types was additive; flies that carried one copy of the *hsp70-Gprk2* construct plus one copy of the *otu-Gprk2-HA* construct laid more eggs than wild type flies. These differences could be due either to dosage (a higher level of expression of *Gprk2* leads to a greater level of egg laying) or to expression in different tissues by the two promoters. Eggs from $gprk2^{6936}$ homozygous females bearing either type of construct had normal dorsal appendages and nurse cells underwent complete cytoplasmic dumping (data not shown). In addition, attempts to induce phenotypes by overexpression of the transgene were unsuccessful. Various heat induction schemes resulted in only small increases in expression that did not alter fertility or ovary morphology. Similarly, we did not detect any differences in

ovary morphology between females with one and two copies of the *otu-Gprk2-HA* construct (data not shown).

When eggs laid by these same females were tested for hatching, we obtained similar results. As shown in Fig. 1C, when the father is wild type, hatching in homozygous *gprk2*^{6936} females is about 46%. In every case, the presence of one copy of the rescue construct in the mother increased hatching to a level that was comparable to wild type. Together these results suggest that expression of wild type *Gprk2* in the germline is sufficient to rescue the sterility of *gprk2*^{6936} and that follicle cell expression is not necessary for egg laying or hatching.

To determine if *Gprk2* expression in the germline is necessary for fertility, we used the FLP/FRT, dominant-sterile method of Chou et al. [11], to generate germline clones of *gprk2*^{6936}. Using lines that carry FRT recombination sites at the base of the third chromosome, recombination between chromosomes carrying *gprk2*^{6936} and an *ovo*^{D1} mutation was induced by expression of FLP recombinase. The resulting egg chambers were either heterozygous for *ovo*^{D1}, and were therefore sterile with a developmental arrest early in oogenesis, or they were homozygous for *gprk2*^{6936}. The *gprk2*^{6936}/*ovo*^{D1} and *gprk2*^{6936}/*gprk2*^{6936} phenotypes were easily distinguishable by ovary morphology.

Females bearing germline clones of *gprk2*^{6936} produced eggs that closely resembled those from homozygous *gprk2*^{6936} females (Fig. 3); eggs were small, cytoplasmic dumping was incomplete, and dorsal appendages were malformed. These results demonstrate that the defects in egg morphology are not due to a loss of expression in the follicle cells, since these defects were observed when the germline was homozygous for *gprk2*^{6936} but the follicle cells were heterozygous. Determinations of egg laying could not be carried out because recombination does not occur in all ovarioles. However, the hatching rate of two independent FRT lines was 12.3% (104/848 hatched) and 7.6% (24/316 hatched) when the males were wild type. These levels are lower than those shown in Fig. 1, however, this value is similar to homozygous *FRT*, *gprk2*^{6936} females (18.7%, 63/337 hatched). The difference is probably due to the fact that the *FRT*, *gprk2*^{6936} stock was not out-crossed because of the possibility of losing the FRT insertion. We know that the *gprk2*^{6936} mutant is sensitive to genetic background and without constant out-crossing, the females become more sterile. These data show that a loss of expression selectively in the germline results in a decrease in fertility.

Maternal and zygotic expression of Gprk2 both contribute to fertility

The results described above demonstrate that expression of *Gprk2* in the germline plays an important role in development of egg chambers and hatching of embryos. However, these experiments did not address the role of zygotic expression in hatching. In the absence of *Gprk2* activity in both the female and the male, the

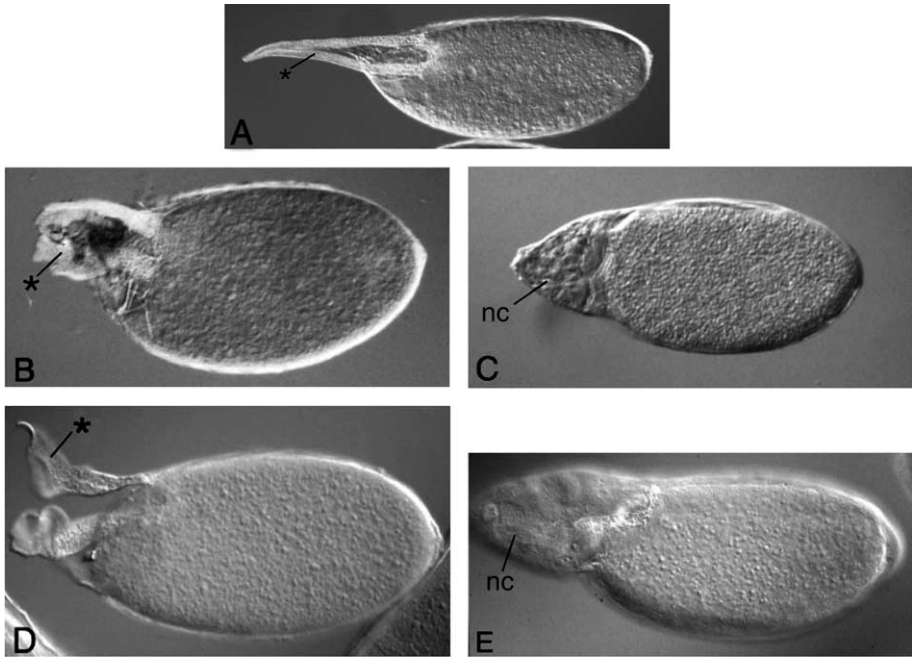

Fig. 3. Germline clones of *gprk2*^{6936} produce eggs that resemble eggs from homozygous *gprk2*^{6936} mothers. (A) In eggs from wild type mothers, the dorsal appendages (*) are long and narrow, and no nurse cells (nc) remain associated with the oocyte. (B,C) Homozygous *gprk2*^{6936} mothers produce eggs with malformed dorsal appendages and with nurse cells that have not completed cytoplasmic dumping, pointed out by nurse cells (nc). (D,E) Females bearing germline clones of *gprk2*^{6936} produce eggs that have the same defects in dorsal appendage formation and cytoplasmic dumping. All eggs are shown at the same magnification.

hatching rate is very low (4%; Fig. 4). When homozygous *gprk2*^{6936} females are mated to wild type males the hatching rate is 46%. This suggests that there is paternal rescue but that it cannot completely compensate for a loss of maternal expression. In contrast, when females are wild type and males are homozygous for *gprk2*^{6936}, the hatching rate is quite high (84%). This presumably represents the role of both maternal and zygotic expression. To compare the role of maternal versus zygotic function in another way, we compared the ability of the *hsp70-Gprk2*, *otu-Gprk2*, and *otu-Gprk2-HA* constructs to rescue hatching when present in the female versus the male.

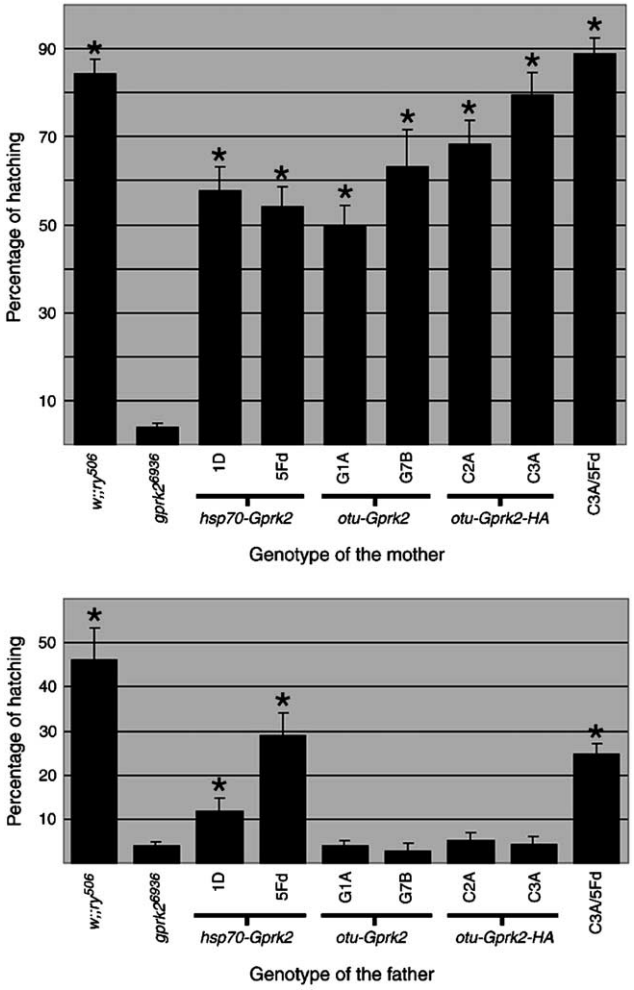

Fig. 4. Comparison of rescue with constructs carried by the mother or the father. (A) Percentage of hatching from crosses in which both males and females were homozygous for *gprk2*^{6936} and the mother carried a single copy of the rescue construct listed at the bottom. (B) Percentage of hatching from crosses in which both males and females were homozygous for *gprk2*^{6936} and the father carried a single copy of the rescue construct listed at the bottom. Asterisks indicate values that are significantly different ($P < 0.05$) from hatching in eggs with no rescue construct (second bar). A comparison of the two graphs demonstrates the difference between maternal plus zygotic expression of *Gprk2* and zygotic expression alone.

When *gprk2*^{6936} females carrying one copy of any of the rescue constructs were mated to a homozygous *gprk2*^{6936} male, all of the constructs yielded a high level of hatching (Fig. 4A), similar to the results of crossing wild type females to *gprk2*^{6936} mutant males. The germline-specific constructs rescued slightly better than the *hsp70-Gprk2* constructs, again pointing out the importance of maternal expression. When introduced through the father, the *otu-Gprk2* and *otu-Gprk2-HA* constructs rescued poorly (Fig. 4B). It should be noted, however, that the degree of rescue in this experiment is under-estimated because the males were heterozygous for the transgene (to increase the viability of the males). Therefore, if the males had carried the *otu-Gprk2* insertion on both chromosomes, the expected level of hatching would be 5–10%. The *hsp70-Gprk2* rescued well (12–30%), with an adjusted rescue level of 24–60%. This is similar to the level of rescue observed when *gprk2*^{6936} females were mated to wild type males. The males carrying both a copy of the *otu-Gprk2-HA* transgene and a copy of the *hsp70-Gprk2* transgene rescued to the same degree as the *hsp70-Gprk2* alone. These results confirm that both maternal and zygotic expression contribute to hatching, in roughly equal degrees. Furthermore, zygotic expression of the *otu*-driven constructs does not play a large role in hatching (Fig. 4B). The high degree of adult viability with the *otu-Gprk2* constructs (Fig. 1A) is likely to have resulted from post-embryonic expression.

Discussion

The ability of *hsp70-Gprk2* and *otu-Gprk2* constructs to rescue adult viability, egg laying, and hatching in the *gprk2*^{6936} mutant demonstrates that the *Gprk2* gene is responsible for the defects observed in this mutant line. Furthermore, it suggests that *Gprk2* activity carries out critical functions, at multiple stages of development, that are not redundant with other GRKs. The GPRK2 protein belongs to the mammalian GRK subgroup that contains GRK4, GRK5, and GRK6, and one *Caenorhabditis elegans* homologue. The other known *Drosophila* GRK, GPRK1, falls into the GRK subgroup that contains GRK2 (βARK1), GRK3 (βARK2), and another *C. elegans* homologue [16–18]. There is no known *Drosophila* homologue for GRK1 (rhodopsin kinase). The non-overlapping functions of *Gprk1* and *Gprk2* could arise from differential expression or localization, or from different receptor specificities. There is no detectable expression of GPRK2 protein in follicle cells or thoracic muscles. These could be tissues that preferentially express GPRK1. The mechanisms that control receptor specificity of GRKs have not been determined but mouse transgenic studies have shown that such specificities do exist. Mice overexpressing GRK5 in

the heart show increased desensitization of β-adrenergic receptors but no change in the response of angiotensin II receptors. In contrast, mice overexpressing GRK3 show the opposite response [5,19]. Therefore, there is precedent for different GRKs carrying out distinct functions.

Follicle cell expression of Gprk2 is not necessary for fertility

The follicle cells play little or no role in the function of *Gprk2*. This is supported by the fact that germline-specific expression of the *Gprk2* gene rescues egg laying, and lack of expression in the germline mimics the ovary morphology of the *gprk2*^{6936} homozygote and reduces hatching rates. This supports our earlier findings that there is no *Gprk2* mRNA or protein expression in the follicle cells. This suggests that the disruption of dorsal appendage formation in the *gprk2*^{6936} mutant is secondary to defects in the nurse cells. This could occur in a number of ways. First, the migratory path that the follicle cells must follow to form the dorsal appendages could be absent or altered because of the lack of *Gprk2* expression. Another possibility is that the nurse cells that fail to complete cytoplasmic dumping form a physical barrier to the formation of dorsal appendages. This theory is supported by the existence of many mutants that disrupt both cytoplasmic dumping and dorsal appendage formation, such as *chickadee*, *singed*, *quail*, and *bullwinkle* [20–23].

Maternal and zygotic contributions play an equal role in hatching

The hatching studies described above demonstrate that both maternal and zygotic expression of *Gprk2* are important for fertility, as measured by hatching rates. The maternal contribution is seen best in the ability of *otu-Gprk2* constructs to rescue hatching when the father is homozygous for *gprk2*^{6936} (Fig. 4A). This rescue is due, in part, to zygotic expression of *otu-Gprk2*, however, Fig. 4B shows that this contribution is rather low. In crosses where the males are homozygous for *gprk2*^{6936} and females carry an *otu-Gprk2* construct, hatching ranges from 50% to 80%. In contrast, in crosses where females are homozygous for *gprk2*^{6936} and males carry one copy of an *otu-Gprk2* construct, hatching rates are about 2.6–5.0% (or an adjusted rate of about 5–10%). Thus, the maternal expression of *Gprk2* plays an important role in hatching.

The paternal contribution can be seen in crosses of homozygous *gprk2*^{6936} females to wild type males or males carrying an *hsp70-Gprk2* construct. In the first case, hatching is about 50% and in the second case, adjusted rates range from 24% to 60%. Although these hatching rates are lower than when females carry a wild type copy of *Gprk2*, they do demonstrate that oogenesis and early embryogenesis can proceed without detectable germline expression of *Gprk2*. It is important to remember, however, that few eggs develop in this way because females that are homozygous for *gprk2*^{6936} lay fewer eggs. These results suggest that *Gprk2* plays a role both in early and late embryogenesis. This is consistent with analyses of the embryos from homozygous *gprk2*^{6936} mothers that failed to hatch. These embryos had defects throughout embryogenesis, suggesting functions at different developmental times [7].

What are the targets of GPRK2 in the ovaries?

GRKs have been shown to phosphorylate both receptors and non-receptor targets. If GPRK2 protein phosphorylates a receptor, our localization and rescue studies would suggest that the receptor is expressed in the membrane of the nurse cells and/or the oocyte, although candidate receptors have yet to be identified. If the receptor ligand is soluble it could come from the ovary or from extra-ovarian tissues. An extra-ovarian ligand (for example, a peptide) probably would not have access to the egg chamber before stage 8 when pathways open between the follicle cells to allow the passage of yolk proteins. This would be consistent with the developmental timing of the *gprk2*^{6936} phenotype; the majority of defects appear after stage 8. *Gprk2* could be involved in signal pathways that are initiated by soluble ligands released from the follicle cells or oocyte. Examples of this type of signaling are seen in embryogenesis with the torso-like ligand that interacts with the torso receptor to pattern the ends of the egg, or the Spaetzle zymogen which activates the Dorsal protein to determine the dorsal–ventral axis of the embryo. Both of these ligands are present in the perivitelline space between the follicle cells and the oocyte [24–26]. GPRK2 protein is also localized between nurse cells, suggesting that there could also be receptors that interact with membrane-bound ligands.

Receptors that act through the second messenger, cAMP, are likely candidates for GPRK2 targets. In homozygous *gprk2*^{6936} ovaries, cAMP levels are about threefold lower than in wild type ovaries. In addition, *gprk2*^{6936} interacts genetically with mutants of *dunce*, a cAMP-specific phosphodiesterase. In females that are homozygous for *gprk2*^{6936} and heterozygous for *dunce*, egg laying and hatching are significantly higher than in *gprk2*^{6936} homozygotes and egg morphology is considerably improved [8]. Interestingly, clonal analysis suggested that expression of *dunce* is required in the somatic cells for egg laying [27], although these studies did not rule out a role for *dunce* in the germline.

It is also possible that GPRK2 protein interacts with the cytoskeleton, like mammalian GRK5 [4]. The nurse cell cytoskeleton plays a critical role in cytoplasmic dumping, a process which is disrupted in the *gprk2*^{6936}

mutant. Just prior to the initiation of dumping, actin fibers form and span the cytoplasm from the nuclear membrane to the plasma membrane. These fibers are thought to tether the nurse cell nuclei during cytoplasmic dumping so that they do not block the ring canals or enter the oocyte [20–22]. In a small number of egg chambers from homozygous egg *gprk2*^{6936} chambers (about 30%), we observe a defect in tethering of the nurse cell nuclei, although the cytoplasmic actin fibers appear to be normal [7]. This phenotype is similar to, but much weaker than, mutants that disrupt proteins involved in actin bundling. In the *chickadee*, *quail*, and *singed* mutants (which disrupt homologues of profilin, villin, and fascin, respectively) the cytoplasmic actin fibers fail to form, nurse cell nuclei enter the ring canals, and dumping is blocked completely [20–22]. Perhaps *Gprk2* is not a structural component of the actin fibers but, instead, plays a regulatory role in the process of cytoplasmic dumping.

In summary, these results confirm that the *Gprk2* gene is responsible for the defects observed in the *gprk2*^{6936} mutant and that *Gprk2* plays a role in both maternal and zygotic stages of development. The interaction between *gprk2*^{6936} and *dunce* mutants suggests that one of the major targets of the GPRK2 protein is receptors that modulate cAMP levels. Furthermore, the observation of ring canal defects in the *gprk2*^{6936} mutant raises the possibility of a close association between GPRK2 protein and the cytoskeleton. This association is further suggested by the finding that a mammalian homologue of *Gprk2* (GRK5) can bind to actin and α-actinin [2,28]. This manuscript lays the groundwork for an in vivo analysis of *Gprk2* function in development. The availability of rescue constructs and the delineation of the developmental times at which *Gprk2* function is required introduce the possibility of identifying the substrates that allow GPRK2 to carry out its developmental role.

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