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Control of Germline *torso* Expression by the BTB/POZ Domain Protein Pipsqueak Is Required for Embryonic Terminal Patterning in Drosophila

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ABSTRACT

Early embryogenesis in *Drosophila melanogaster* is controlled by maternal gene products, which are deposited in the egg during oogenesis. It is not well understood how maternal gene expression is controlled during germline development. *pipsqueak* (*psq*) is a complex locus that encodes several nuclear protein variants containing a PSQ DNA-binding domain and a BTB/POZ domain. Psq proteins are thought to regulate germline gene expression through epigenetic silencing. While *psq* was originally identified as a posterior-group gene, we show here a novel role of *psq* in embryonic terminal patterning. We characterized a new *psq* loss-of-function allele, psq^{rum}, which specifically affects signaling by the Torso (Tor) receptor tyrosine kinase (RTK). Using genetic epistasis, gene expression analyses, and rescue experiments, we demonstrate that the sole function impaired by the psq^{rum} mutation in the terminal system is an essential requirement for controlling transcription of the *tor* gene in the germline. In contrast, the expression of several other maternal genes, including those encoding Tor pathway components, is not affected by the mutation. Rescue of the psq^{rum} terminal phenotype does not require the BTB/POZ domain, suggesting that the PSQ DNA-binding domain can function independently of the BTB/POZ domain. Our finding that *tor* expression is subject to dedicated transcriptional regulation suggests that different maternal genes may be regulated by multiple distinct mechanisms, rather than by a general program controlling nurse-cell transcription.

THE early steps of Drosophila embryonic development are under the control of a set of products supplied by the mother to the oocyte. The genes encoding these products are specifically transcribed in the female germline, which consists of cysts of 16 cells, the oocyte plus 15 auxiliary nurse cells. Thereafter, their RNAs or proteins are transported from the nurse cells into the oocyte (reviewed in Spradling 1993). However, while the role of these maternal genes is fundamental for early development, not much is known about how their expression is regulated.

Among those maternal products is a set of determinants responsible for the spatially restricted activation of early zygotic genes, which dictate the broad subdivisions of the future organism. Four different maternal systems are involved in setting up the body pattern of the embryo along the anterior–posterior and dorsoventral axes. One of these, the terminal system, is responsible for the specification of the terminal regions at both poles of the embryo (reviewed in Duffy and Perrimon 1994; Furriols and Casanova 2003). A central element in the terminal system is the product of the *torso* (*tor*) gene, a receptor tyrosine kinase (RTK) (Sprenger *et al.* 1989).

tor is transcribed in the nurse cells and its RNA is deposited in the oocyte, where it is thought to remain untranslated until fertilization. Upon translation, Tor protein accumulates ubiquitously at the blastoderm surface. However, it is activated only at the blastoderm poles by a mechanism that appears to be triggered by the localized cleavage of its ligand, the protein Trunk (Casanova *et al.* 1995; Casali and Casanova 2001). Upon activation, Tor triggers the Ras/Raf/MAPK signaling pathway, which downregulates a repressor complex containing the HMG domain protein Capicua (Cic) and the corepressor Groucho (Gro) (Paroush *et al.* 1997; Jimenez *et al.* 2000). As a result, two zygotic genes, *tailless* (*tll*) and *huckebein* (*hkb*), are specifically expressed at both embryonic poles. Mutations in components of the Tor signaling pathway give rise to embryos lacking the terminal regions (the terminal phenotype), whereas constitutive activation of the pathway leads to the opposite phenotype, in which terminal cell fates expand into central regions of the embryo (Klingler *et al.* 1988; Casanova and Struhl 1989; Furriols and Casanova 2003).

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Not much is known about how the spatiotemporal patterns and levels of expression of the maternal genes are regulated. Here we describe a new role of *pipsqueak* (*psq*) in regulating *tor* transcription. *psq* is a widely expressed gene that by alternative splicing gives rise to several protein variants that share a PSQ DNA-binding motif (Weber *et al.* 1995; Horowitz and Berg 1996; Lehmann *et al.* 1998). Many *psq* mutant alleles have been recovered that show distinct embryonic and adult phenotypes. Our results now show that a particular set of *psq* allelic combinations gives rise to embryos with terminal defects. We demonstrate that these defects are due to a requirement of *psq* for *tor* expression in the germline. The specific transcriptional regulation of *tor* points to multiple and distinct regulatory mechanisms for different maternal genes, rather than a general mechanism for the regulation of nurse-cell transcription.

MATERIALS AND METHODS

Genetics: The mutagenesis screen was previously described (Luschnig *et al.* 2004). The following fly stocks were used and are listed in FlyBase: dec^{1}, *FRTG13* dec^{+}, *hs-Flp*^{122}, psq^{0115}, psq^{8109}, psq^{D91}, psq^{E34}, psq^{E39}, psq^{EP2011}, psq^{F112}, psq^{fs1}, psq^{HK38}, $psq^{KG02404}$, $psq^{KG00811}$, psq^{rev2}, psq^{rev4}, psq^{rev7}, psq^{rev9}, psq^{rev12}, psq^{rev14}, and tor^{Y9}. The psq^{rum} mutation was mapped to the cytological interval 47A–47B14 on the basis of noncomplementation of Df(2R)stan1, Df(2R)stan2, Df(2R)12, Df(2R)E3363, and Df(2R)47A and complementation of Df(2R)X1, Df(2R)X3, and Df(2R)en-A. The tor^{Y9} psq^{rum} chromosome was generated by meiotic recombination. Double mutant psq^{rum}; cic^{1} embryos were generated by inducing *FRT82B* cic^{1} homozygous germline clones (Chou and Perrimon 1996) in psq^{rum} homozygous females. To rescue the psq^{rum} mutation, males carrying either *UASp-psq-1* or *UASp-psq-2* and psq^{rum} were crossed to females carrying psq^{rum} and *mat-α-tub67C-Gal4-VP16* (Hacker and Perrimon 1998). From this cross, females of the genotype *UASp-psq;* psq^{rum}/psq^{rum}*; mat-α-tub67C-Gal4-VP16/+* were collected and their embryonic progeny were analyzed in cuticle preparations. The rescue experiment with *UASp-tor* was done accordingly, except that *nos-Gal4-VP16* (Van Doren *et al.* 1998) was used as a Gal4 driver. Marked psq^{rum} follicle cell clones were generated using the *defective chorion* method (Nilson and Schupbach 1998).

Cuticle preparations: Cuticle preparations were done as previously described (Luschnig *et al.* 2004).

Whole-mount *in situ* hybridization: *In situ* hybridizations were performed using a standard protocol (Tautz and Pfeifle 1989). Digoxigenin–UTP-labeled antisense RNA probes were prepared from *hkb*, *tll*, and *tor* cDNAs. *In situ* hybridizations of wild-type and mutant embryos or ovaries were developed in parallel.

Antibody stainings: Stainings were performed using standard protocols. The following antibodies were used: rabbit anti-Cic (Jimenez *et al.* 2000), mouse anti-HA (12CA5; Roche), mouse anti-dpERK (Cell Signaling Technologies), anti-β-galactosidase (559761; Cappel), and rabbit anti-Vasa (Gilboa and Lehmann 2004). Secondary antibodies were anti-rabbit Cy2, anti-mouse Cy3, and anti-rabbit-Cy5 at 1/300 (Jackson ImmunoResearch).

Microscopy: Dark field photographs were taken using a Nikon Eclipse 80i microsocope with a Nikon digital camera DXM 1200F. Confocal images were obtained with a Leica SPE or an Olympus FV 1000 microscope.

Molecular characterization of the psq^{rum} mutation: Coding exons, exon–intron junctions, and 5′- and 3′-UTRs of the *psq* gene were sequenced in genomic DNA from psq^{rum} homozygous flies and from flies carrying the parental chromosome (*FRTG13 c px sp*; Luschnig *et al.* 2004). Fragments of 400–600 bp were amplified and sequenced using various primers to obtain full multiple coverage of each fragment. Total RNA from mutant and wild-type ovaries was isolated using Trizol (Invitrogen). cDNA was synthesized using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas), using both random hexamer primers and oligo-dT primers. We amplified cDNA from wild-type and mutant ovaries (and genomic DNA as a control) using primers located in the exons flanking the mutation. Fragments were purified, cloned into pGEM-T (Promega), and sequenced.

Real-time PCR: Real-time PCR was performed with a Roche LightCycler 480 using the SYBR Green I method according to the manufacturer's instructions. Primers for *tor*, *grk*, *ras*, *fs(1)ph*, and for the *rp49*, *alpha-Tubulin-84B*, and *act5C controls* were designed on exon–exon junctions using Primer Express software (Applied Biosystems). We extracted total RNA from ovaries and prepared cDNAs using random hexamer primers. The nature of the PCR products was confirmed by melting curve analysis. All analyses were performed using the Relative Expression software tool (Pfaffl *et al.* 2002). Main steps of the automatic REST workflow are as follows: PCR efficiencies were calculated for every pair of primers by generating standard curves at increasing dilutions of cDNA (1:1, 1:5, 1:25, 1:125, and 1:625) and were used to correct raw data. *rp49*, *alpha-tubulin-84B*, and *act5C* were assumed to be equally expressed in wild-type and mutant ovaries and were used for normalization. A ratio between the normalized signals of tested genes in mutant and wild type was calculated and expressed as fold increase/decrease and statistically tested by a bootstrap test (10,000 randomizations).

Transgenic constructs: UASp-*psq-1* was generated by inserting the *psq-1* cDNA (including 5′- and 3′-UTR sequences) as a *Not*I fragment from pHPT7-9 (Horowitz and Berg 1996) into pUASp (Rorth 1998). UASp-*psq-2* was generated by amplifying the coding sequence of Psq-2 (corresponding to amino acids 420–1061 of Psq-1) from UASp-*psq-1* using oligonucleotides Psq2-*Not*I-For (5′-tataGCGGCCGCatgactagtt taggcatgg) and pUASp-Rev (5′-tcaagctcctcgagttaacg). The PCR fragment was cut with *Not*I and inserted into pUASp. The resulting fragment retains the *psq* 3′-UTR, but lacks *psq* 5′-UTR sequences. The PCR-amplified part of the construct was confirmed by DNA sequencing. The *tor*–LacZ construct was generated by inserting a *Not*I genomic fragment of 3.5 kb from the *tor* promoter described in Furriols *et al.* (1998) into the C4PLZ transformation vector (Wharton and Crews 1993). UASp-*tor* was generated by cloning the *tor* cDNA (RE49094; DGRC) into pUASp. Transgenic flies were generated by *P*-element transformation. At least two independent insertions were analyzed for each construct.

RESULTS

***rumpf* is a new factor involved in Tor signaling:** In a mutagenesis screen for new maternal genes (Luschnig *et al.* 2004) we found that embryos from *2R-225-5* homozygous mutant germline clones lacked cuticle structures derived from the anterior and posterior poles of the embryo (Figure 1, A and B), resembling the phenotype of terminal-class mutations. On the basis of

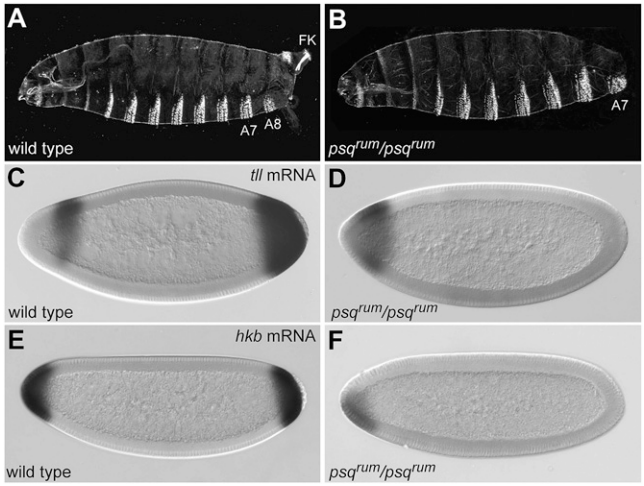

FIGURE 1.—*psq*^{rum} impairs embryonic terminal development. (A and B) Cuticle phenotype of embryos from wild-type and *psq*^{rum} homozygous females. *psq*^{rum} embryos lack terminal structures (A8, abdominal segment 8; FK, Filzkörper). (C–F) Expression patterns of *tailless* (*tll*; C and D) and *huckebein* (*hkb*; E and F) in wild-type (C and E) and *psq*^{rum} (D and F) early embryos. Expression patterns in *psq*^{rum} mutants are reminiscent of those in embryos lacking Tor signaling.

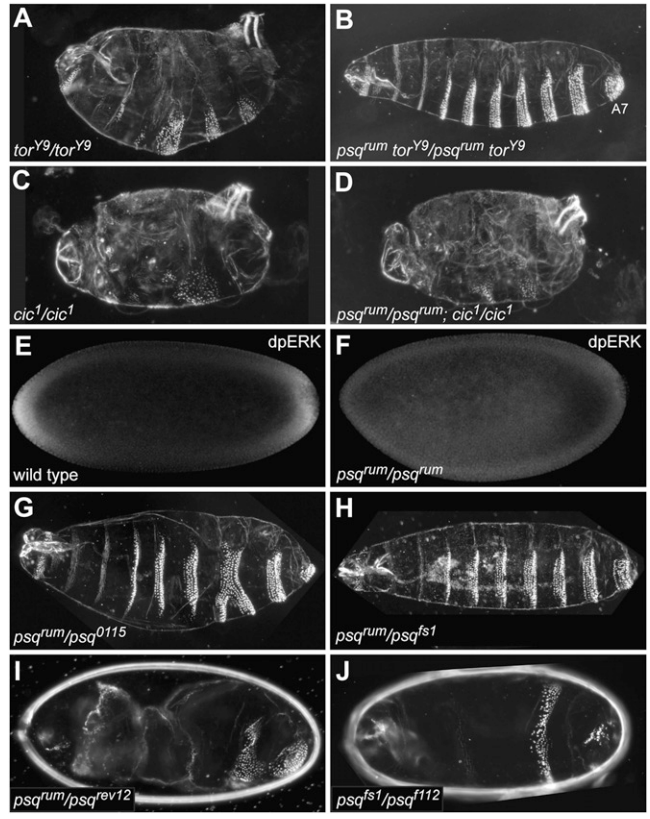

FIGURE 2.—*psq*^{rum} acts upstream of MAPK and downstream of or at the level of the Tor receptor. (A) Embryos from *tor*^{Y9} homozygous females display ectopic activation of the Tor receptor as indicated by the deletion of several abdominal segments. (B) In contrast, embryos from *tor*^{Y9} *psq*^{rum} double mutant females show terminal defects resembling the *psq*^{rum} single mutant phenotype. (C) Embryos from *cic*^{1} homozygous females show a *tor* gain-of-function phenotype. (D) This phenotype is not modified in embryos from *psq*^{rum}; *cic*^{1} homozygous germline clones. (E) MAPK activation is detected at the poles in wild-type embryos by a specific antibody that recognizes its diphosphorylated form (dpERK). (F) Such activation is not detected in *psq*^{rum} mutants. (G–I) Heteroallelic combinations of *psq*^{rum} with other *psq* alleles also give rise to terminal phenotypes. (J) A combination of other *psq* alleles besides *psq*^{rum} also produces embryos showing terminal defects in addition to a lack of most abdominal segments.

this phenotype we named the locus *rumpf* (*rum*; German for "torso"). Homozygous *rum* flies show no obvious defects, but homozygous females produce embryos with terminal defects resembling those of *rum* germline clone-derived embryos. Although the cuticle phenotype of embryos lacking maternal *rum* function (from here on referred to as *rum* embryos) was somewhat variable and often showed deletions of one or more abdominal segments, all embryos completely lacked terminal structures ($n = 100$). The development of these structures depends on the terminal gap genes *tll* and *hkb*, whose expression at the posterior pole is solely dependent on Tor signaling. Posterior expression of *tll* and *hkb* was abolished in *rum* embryos, reminiscent of the phenotype of mutants lacking Tor signaling (Figure 1, C–F). These results indicate that activity of the Tor signaling pathway is severely compromised or completely abolished in *rum* embryos.

***rum* is required for neither Tor receptor activation nor *tll* and *hkb* transcription:** We asked at which level *rum* is interfering with the Tor pathway. The function of *rum* could be in either generating the terminally restricted signal in the somatic follicle cells or the transmission or interpretation of the signal. However, the germline-specific requirement excludes that the *rum* mutation affects a follicle cell function needed for Tor signaling. Consistent with this notion, large clones of *rum* mutant follicle cells do not affect terminal patterning of the embryo (supporting information, Figure S1). We asked whether *rum* acts upstream or downstream of the Tor RTK by testing epistatic relationships between *rum* and *tor*. Embryos from homozygous females carrying the *tor* gain-of-function mutation *tor*^{Y9} show expanded terminal regions at the expense of central body regions, as indicated by the deletion of several abdominal segments (Figure 2A). In contrast, all embryos from homozygous *tor*^{Y9} *rum* double mutant females show terminal defects resembling the *rum* single mutant phenotype (Figure 2B; $n = 50$). Thus, *rum* is epistatic to *tor*^{Y9}.

In the absence of Tor signaling, a repressor complex containing Cic inhibits *tll* and *hkb* expression. In the absence of *cic* function, *tll* and *hkb* are derepressed and their respective expression domains extend into the middle of the embryo, resulting in a *tor* gain-of-function phenotype (Figure 2C; JIMENEZ *et al.* 2000). We observed the same *tor* gain-of-function phenotype in all embryos derived from *rum; cic*^{1} double mutant germline

clones (Figure 2D; $n = 40$). Thus, *cic* is epistatic to *rum*. Finally, while MAPK/ERK phosphorylation is detected at the poles in the wild type (Gabay *et al.* 1997), it is not detected in *rum* embryos (Figure 2, E and F). Consequently, degradation of Cic protein at the poles is also abolished in *rum* embryos (data not shown). Altogether, these results indicate that *rum* acts upstream of MAPK and downstream of or at the level of the Tor receptor.

***rum* is a mutation in the *psq* locus:** We mapped the *rum* mutation to the cytological interval 47A–47B14. Within this interval, two *P*-element insertions, EP(2)2011 and KG2404, failed to complement female sterility of the *rum* mutation. The two *P*-elements are inserted in the 48-kb spanning intron 3 of the *psq* gene (Figure 3A; Spradling *et al.* 1999; Bellen *et al.* 2004). *psq* is a large and complex locus, which, through the use of three alternative promoters and alternative splicing, gives rise to at least 12 different transcripts (FlyBase; Weber *et al.* 1995; Horowitz and Berg 1996; Ferres-Marco *et al.* 2006). The *psq* locus was originally identified on the basis of its maternal-effect posterior group phenotype (Schupbach and Wieschaus 1991; Siegel *et al.* 1993). However, several additional *psq* alleles show distinct embryonic or adult phenotypes. *psq* mutations include lethal alleles (0482; Horowitz and Berg 1996), semilethal alleles that cause rudimentary ovaries and a defect in R3/R4 photoreceptor specification (F112, E34, E39, D91; Weber *et al.* 1995), and viable female-sterile alleles causing defects in abdominal and dorsoventral patterning and in pole cell formation (fs1, I-30, HK38, 2403, 2905, 6372, P1, P4, and X1-30; Schupbach and Wieschaus 1991; Siegel *et al.* 1993; Horowitz and Berg 1996). However, a role of *psq* in terminal patterning has not been described. Complementation tests between *rum* and other *psq* alleles (summarized in Table S1) revealed complex interactions. Heteroallelic combinations between four *psq* alleles (fs1, 0115, KG02404, and rev12) and the *rum* mutation gave rise to embryonic terminal defects of variable strength and penetrance (Figure 2, G–I; Table S1). Interestingly, certain allelic combinations give rise to embryos that arrest early in development, while other alleles complement female sterility of the *rum* mutation. In addition, we found that embryos from transheterozygous females carrying a combination of two other recessive *psq* mutations (psq^{fs1}/psq^{F112}) also show terminal defects (Figure 2J). The complex genetic behavior of *psq* mutations suggests that the different Psq isoforms might mediate distinct functions during oogenesis and embryogenesis and that *rum* is a new *psq* allele that affects a requirement of *psq* in the Tor pathway.

Finally, to conclude whether the phenotype associated with the *rum* mutation is due to a loss of *psq* function, we generated transgenic flies expressing the longest *psq* transcript variant (*psq*-1; Figure 3; Horowitz and Berg 1996). Expression of this construct in the germline of *rum* females rescued the *rum* terminal defects in embryos (Figure 3, C and D). The majority (90%, $n = 200$) of the embryos hatched and developed into fertile adults. These results indicate that the terminal defects in *rum* embryos are due to a requirement of *psq* in the germline. We therefore refer to the *rum* mutation as psq^{rum} from here on.

***psq^{rum}* impairs pole cell formation:** *psq* was proposed to regulate posterior patterning and pole plasm formation through promoting the expression of Vasa (Vas) protein (Siegel *et al.* 1993). As for other *psq* alleles, psq^{rum} embryos showed a severely reduced number of primordial germ cells (pole cells) (Figure 4). Wild-type embryos had on average 29.6 ± 3.75 ($n = 15$) Vas-positive pole cells, whereas psq^{rum} embryos had only 7.4 ± 5.75 ($n = 27$) pole cells, with some embryos completely lacking pole cells. However, the mild posterior-patterning defects in psq^{rum} embryos suggest that *vas* function in the mutants is nearly sufficient for normal abdominal patterning.

Lack of terminal structures and pole cells in psq^{rum} can be rescued by a Psq isoform lacking the BTB/POZ domain: The *psq* locus encodes two major classes of proteins with different N termini (Figure 3B; Horowitz and Berg 1996). The long isoform Psq-1 (1046 aa) contains an N-terminal BTB/POZ domain and four repeats of the PSQ helix-turn-helix DNA-binding motif (Schwendemann and Lehmann 2002; Siegmund and Lehmann 2002). While the BTB/POZ domain provides a platform for protein–protein interactions (Perez-Torrado *et al.* 2006), the Psq repeats mediate binding to a GAGAG consensus DNA motif *in vitro* (Lehmann *et al.* 1998; Siegmund and Lehmann 2002). The short isoform Psq-2 (646 aa) is encoded by *psq-2* and *psq-3* class transcripts and lacks the N-terminal part of the protein harboring the BTB/POZ domain. However, the Psq repeats are common to all known Psq isoforms. To address whether Psq-1 and Psq-2 isoforms exhibit distinct functions with respect to Tor signaling, we generated a UAS-*psq-2* transgene lacking the BTB/POZ domain. Germline-specific expression of this construct rescued the terminal defects in 49% ($n = 190$) of psq^{rum} embryos (Figure 3E). While these embryos hatched and were morphologically normal, terminal patterning in the remaining unhatched embryos (51%, $n = 190$) was only partially rescued (data not shown). We cannot exclude that the partial rescuing activity of *psq-2* transgenes may be due to differences in expression levels between UAS-*psq-1* and UAS-*psq-2* constructs. However, maternal expression of either UAS-*psq-1* or UAS-*psq-2* restored pole cell formation in psq^{rum} embryos (Figure 4E). These findings suggest that the BTB/POZ domain is not strictly required for *psq* function in terminal signaling and pole cell formation. However, we cannot rule out that the transgenic Psq-2 protein might interact with and stabilize residual wild-type Psq proteins in psq^{rum} mutants and thereby mediate the rescue in an indirect fashion.

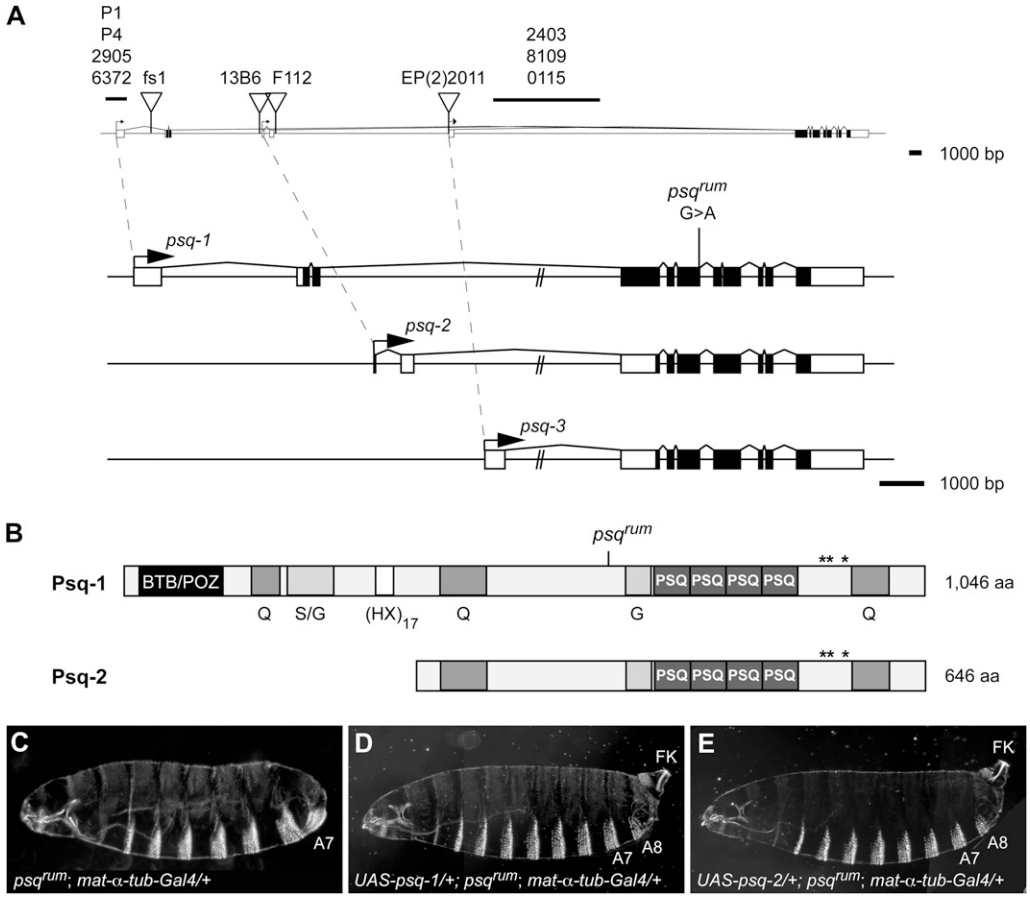

FIGURE 3.—*psq*^{rum} is a mutation in the *psq* locus. (A) Genomic organization of the *psq* locus with *P*-element insertions indicated by triangles, or by horizontal bars for approximate positions, are shown on top. The three major classes of *psq* transcripts originating from the three alternative promoters are shown below. Coding exons are shown as solid boxes; noncoding exons and untranslated regions are shown as open boxes. The splice site mutation in *psq*^{rum} is indicated. (B) Domain structure of two main Psq protein isoforms, Psq-1 and Psq-2. Note that only the long isoform Psq-1 contains the BTB/POZ domain, whereas both long and short isoforms contain the PSQ DNA-binding domain. Regions rich in glutamine (Q), glycine (G), or serine and glycine (S/G), and a repeat of 17 dipeptides of the structure (HX)17 are indicated. Asterisks denote potential MAPK phosphorylation sites (PXT/SP). The *psq*^{rum} mutation is predicted to result in truncation of all Psq protein isoforms at the position marked by the vertical line above the Psq-1 protein. (C–E) Germline-specific expression of *psq* cDNAs rescues the terminal defects of *psq*^{rum} embryos. A control *psq*^{rum} embryo (C) lacks terminal structures, whereas expression of either *psq-1* (D) or *psq-2* (E) under the control of maternal Gal4 restores the formation of terminal structures. Note that maternal expression of *psq-1* in *psq*^{rum} embryos yielded a hatch rate of 90% ($n = 200$), while *psq-2* expression yielded a hatch rate of 49% ($n = 190$). The unhatched embryos show variable terminal defects (data not shown).

The *psq*^{rum} mutation alters the proper splicing of *psq* mRNAs: To identify the molecular lesion that causes the *psq*^{rum} mutation, we sequenced the annotated *psq* coding region, 5′- and 3′-untranslated regions (UTRs), and the reported intron/exon boundaries. We did not detect any change in the coding sequence and the putative regulatory regions analyzed. However, we found a G-to-A transition affecting a splice donor site at the junction between exon 6 and the following intron. By sequencing RT–PCR-amplified cDNA fragments, we confirmed that splicing at this site occurs in wild-type ovaries (Figure 5). The splicing is abnormal in *psq*^{rum} mutants, as a new transcript is produced that escapes splicing and retains the mutated intron. However, splicing is not absolutely impaired in the mutant, as the wild-type variant is also detected (Figure 5). The new reading frame in the abnormal transcripts contains a stop codon 16 aa downstream of the nonspliced exon/intron junction. If translated, the resulting Psq proteins would be truncated and would lack the entire DNA-binding domain. Importantly, as all known *psq* transcripts share the mutated region, the mutation is likely to affect all Psq protein isoforms. Although the truncated Psq proteins could act in a dominant negative fashion, we consider more likely and consistent with the genetic data that *psq*^{rum} is a loss-of-function mutation (see DISCUSSION). Thus, the *psq*^{rum} phenotype appears to be caused by a reduction of the normal levels of one or more of the wild-type *psq* transcripts due to the aberrant splicing.

***psq* is required for normal expression of *tor* mRNA:** Since the *psq* locus encodes nuclear DNA-binding proteins, *psq* could influence Tor signaling by regulating the expression of one or more components of the pathway. On the basis of our genetic results, *psq* could be required for the expression of any of the genes that encode components acting between and including the Tor receptor and MAPK, *i.e.*, Tor, Sos, Ras1, Raf, Dsor, and MAPK. The adaptor proteins Drk, Dos, and Dshc are less likely candidates, since mutation of any one of the corresponding genes does not lead to a complete loss of signaling activity, as observed in *psq*^{rum} embryos (LUSCHNIG *et al.* 2000). We measured steady-state

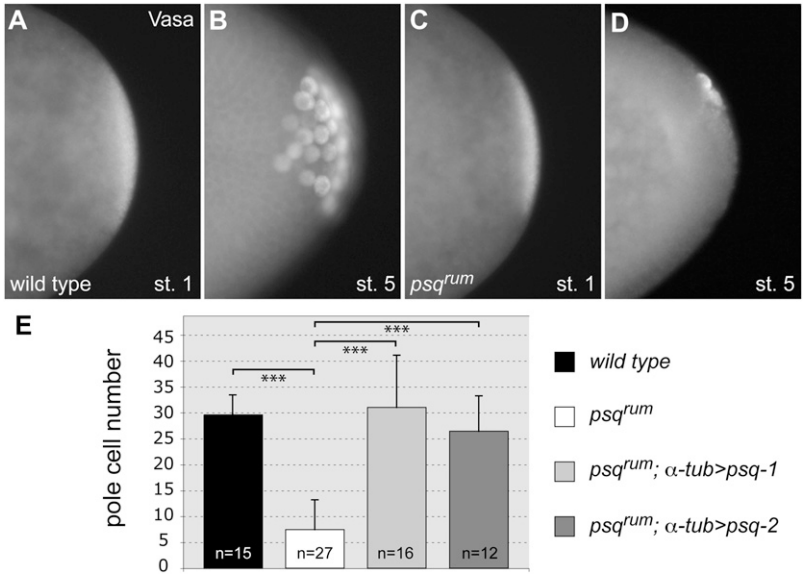

FIGURE 4.—*psq*^{rum} is required for pole cell formation. (A–D) Immunostaining with an anti-Vas antibody in embryos from wild-type (A and B) and *psq*^{rum} (C and D) females. While Vas levels in early *psq*^{rum} mutants appear similar to those of wild-type embryos, the number of pole cells is strongly reduced. (E) Quantification of reduction of pole cells in *psq*^{rum} mutants. Expression of either *psq-1* or *psq-2* in the maternal germline restores pole cell formation in *psq*^{rum} embryos. Mean pole cell numbers are not significantly different between *psq-1* and *psq-2* expressing *psq*^{rum} embryos, as indicated by Student's *t*-test ($P > 0.1$). Error bars represent mean + SD. ***$P < 0.001$.

transcript levels of *ras1*, *raf*, and *dsor* in wild-type and in *psq*^{rum} ovaries by semiquantitative RT–PCR and found that they are detectable at apparently normal levels (data not shown). To analyze *tor* mRNA levels we performed quantitative real-time RT–PCR and found a strong reduction of *tor* mRNA levels in *psq*^{rum} ovaries (46.7-fold mean reduction; $P < 0.01$; Figure 6G). Consistent with these results, strongly reduced *tor* mRNA levels were detected by *in situ* hybridization in *psq* embryos (*psq*^{rum} and *psq*^{fs1}/*psq*^{F112}; Figure 6, A and B; Figure S2) and ovaries (*psq*^{fs1}/*psq*^{0115}; *psq*^{rev2}/*psq*^{0297}; and *psq*^{rum}/*psq*^{rev4}; data not shown). Conversely, transcript levels of *gurken*, *fs(1)polehole*, and *ras1* were not significantly different between wild-type and *psq*^{rum} ovaries (Figure 6G).

A further confirmation that Tor is the critical component of the pathway affected in *psq*^{rum} mutants comes from the observation that *tor* expression driven by the Gal4/UAS system (Brand and Perrimon 1993) is sufficient to rescue the terminal phenotype of *psq*^{rum} mutants (Figure 6, C and D). Importantly, this indicates that none of the remaining signaling components downstream of the Tor receptor are significantly impaired in *psq*^{rum} mutants. Finally, to support that *psq*^{rum} alters *tor* at the transcriptional level, we analyzed the expression of a *lacZ* reporter gene under the control of the *tor* promoter. While the reporter is clearly detected in the nurse cells in wild-type ovaries, no signal was detected in ovaries from *psq*^{rum} females (Figure 6, E and F). Altogether, these data point to a specific and essential role of *psq* in the regulation of *tor* transcription during oogenesis.

DISCUSSION

We report here a particular role of *psq* in the regulation of *tor* transcription in the Drosophila germline. However, only some *psq* mutations show a terminal phenotype. In addition, these results also highlight a distinct mode of transcriptional control of the *tor* gene, different from other maternal genes, including those that encode other members of the Tor signaling pathway. Below we discuss these issues and their implications.

The role of *psq* in *tor* transcriptional regulation: *psq* has functions in many developmental stages and presumptive null mutants are lethal (Weber *et al.* 1995; Horowitz and Berg 1996). Here, we report that a set of *psq* mutations unveils a specific role in *tor* transcription. Why is this phenotype only observed associated with these particular *psq* mutations? Among the *psq* alleles that allow adult survival, strong mutations block oogenesis at early stages (Siegel *et al.* 1993; Weber *et al.* 1995; Horowitz and Berg 1996). Thus, in those cases, an early requirement in oogenesis would mask a later requirement for *tor* transcription. Psq proteins are present in multiple isoforms (Weber *et al.* 1995; Horowitz and Berg 1996; Ferres-Marco *et al.* 2006). Only a few *psq* alleles have been molecularly characterized and among those many are due to transposon insertions (Weber *et al.* 1995; Horowitz and Berg 1996; Spradling *et al.* 1999; Schwendemann and Lehmann 2002; Thibault *et al.* 2004). Therefore, it is difficult on the basis of the molecular analysis of the *psq* mutations to assign distinct functions to the isoforms generated by the different transcripts.

A number of reasons argue for the *psq*^{rum} mutation unveiling a physiological function of *psq* in *tor* transcription, rather than the *rum* phenotype being a neomorphic effect caused by a special truncated Psq protein. First, the terminal phenotype of the *psq*^{rum} mutation is observed in homozygosity, as well as in *trans*-heterozygous combinations with several other *psq* loss-of-function alleles. Second, the terminal phenotype of the *psq*^{rum} mutation arises in association with a mild

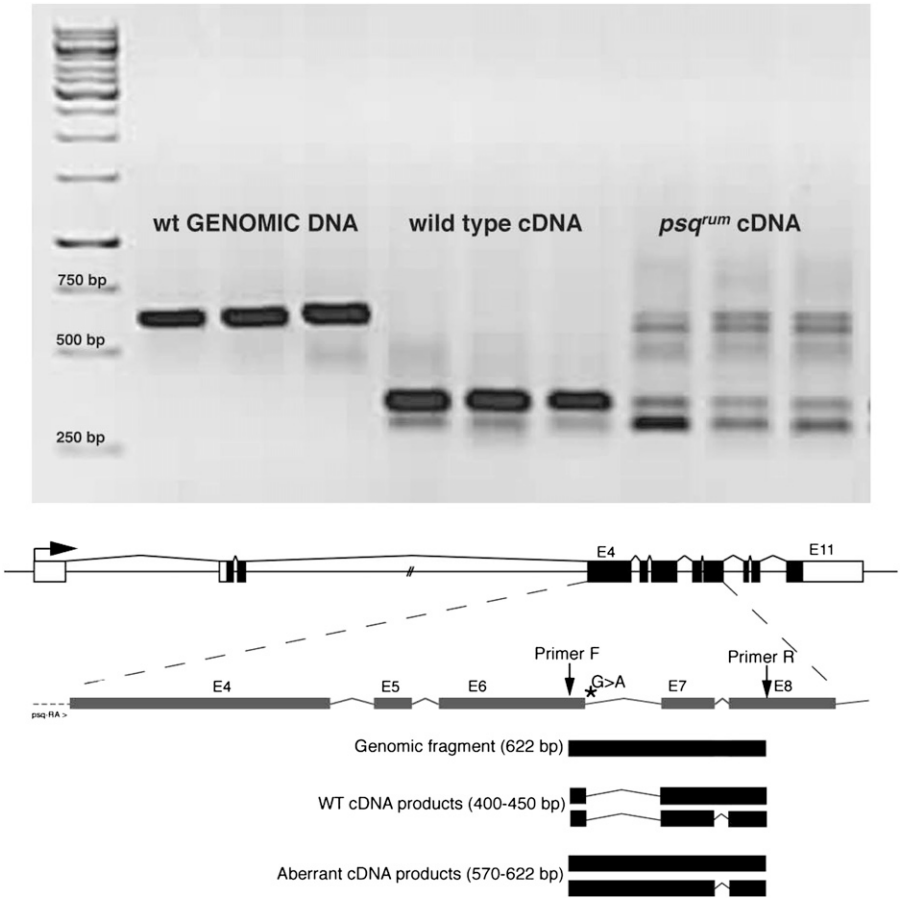

FIGURE 5.—The *psq*^{rum} mutation alters the proper splicing of *psq* mRNAs. Products from three independent PCRs from wild-type genomic DNA and RT–PCR from wild-type and *psq*^{rum} ovaries with primers F and R at exons 6 and 8 of the *psq* gene. A genomic fragment of 622 bp and two cDNA fragments of ~450 bp are detected in the wild type. Additional cDNA fragments of ~600 bp are obtained from *psq*^{rum} mutants. The diagram indicates the organization of the *psq* gene and the PCR products expected from amplification with primers F and R in conditions of normal and aberrant splicing. The point mutation in *psq*^{rum} is indicated by an asterisk. The G in the splice donor site of the intron following exon 6 (in boldface type in the following sequence: CATATGG**g**tgagttg; intron sequence is lowercase) is mutated to A. Numbering of exons corresponds to their appearance in the *psq–RA* transcript variant (FlyBase).

posterior phenotype, a well-known *psq* loss-of-function phenotype. Third, a transheterozygous combination of other *psq* loss-of-function alleles also produces embryos showing reduced *tor* expression and terminal defects. And finally, expression of *psq* rescues the *psq*^{rum} terminal phenotype.

The *psq*^{rum} mutation causes a decrease in the wild-type splicing at one specific site. Nevertheless, since all known isoforms share this splicing, we cannot infer whether a particular isoform is responsible for *tor* transcriptional regulation. However, the rescue experiments indicate that both a long Psq isoform containing the BTB/POZ domain and a short isoform lacking this domain are capable of providing the *psq* function controlling *tor* transcription that is missing in *psq*^{rum} mutants. Thus, on the one hand, the BTB/POZ domain appears to be dispensable for this *psq* function, but on the other hand, a long isoform can substitute for a short isoform, arguing against separate functions of these *psq* isoforms in the context of Tor signaling. Thus, an overall decrease of many *psq* isoforms in the *psq*^{rum} mutant could be affecting *tor* transcription.

In this regard, it is worth considering together the terminal and posterior defects associated with the *psq*^{rum} mutation. *tor* is affected more strongly than *vas* in *psq*^{rum} mutants, while the opposite is true for other *psq* mutants (*e.g.*, *psq*^{HK38}, *psq*^{2403}, and *psq*^{fs1}; SCHUPBACH and WIESCHAUS 1991; SIEGEL *et al.* 1993; HOROWITZ and BERG 1996), in which *vas* is strongly affected, but not *tor*, according to their cuticle phenotype. These data argue against a simple model in which *tor* and *vas* transcription would be impaired below different thresholds of *psq* activity.

As Psq is thought to repress gene expression through epigenetic silencing (HUANG *et al.* 2002; FERRES-MARCO *et al.* 2006), Psq could activate *tor* expression indirectly, through the repression of a still unidentified *tor* repressor. Alternatively, Psq could activate *tor* expression directly. Indeed, genetic interaction studies suggest that *psq* and *Trithorax-like* (*Trl*) act together in transcriptional activation as well as in transcriptional silencing of homeotic genes (SCHWENDEMANN and LEHMANN 2002).

Distinct regulation of *tor* transcription in the germline: Not much is known about how transcription is regulated in Drosophila nurse cells. One possibility is that spatially and temporally coexpressed genes share a common mode of transcriptional regulation. Indeed, enrichment of specific core motifs in the promoters of genes with female germline expression (FITZGERALD *et al.* 2006) is consistent with such a hypothesis. The multiple effects of *psq* mutations during oogenesis might argue for such a general role of *psq*. However, and in spite of *psq*'s multiple requirements in the germline, *tor* transcription appears to be distinctly regulated.

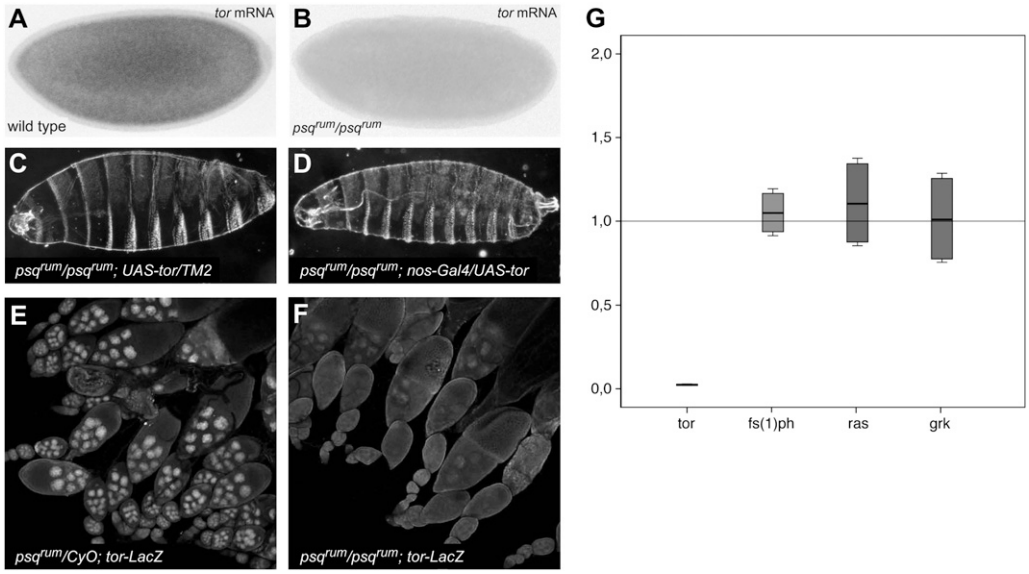

FIGURE 6.—*psq*^{rum} is required for normal expression of *tor* in the germline. (A and B) *tor in situ* hybridization in wild-type embryo (A) and *psq*^{rum} mutant (B). In *psq*^{rum} mutants, *tor* mRNA levels are strongly reduced. (C and D) GAL4-driven expression of *tor* is sufficient to rescue the terminal phenotype in a *psq*^{rum} homozygous background. (E) A *tor–lacZ* construct gives rise to LacZ protein in the nurse cells (note nuclear accumulation because of a nuclear localization signal in the construct). (F) LacZ accumulation from the *tor–lacZ* construct is abolished in homozygous *psq*^{rum} ovaries. (G) *tor* mRNA is 46.7-fold reduced in *psq*^{rum} ovaries compared to the wild type, as detected by real-time PCR. In contrast, mRNA levels of *fs(1)ph*, *ras*, and *grk* are not affected. The *y*-axis indicates relative expression levels as fold change between wild-type and *psq*^{rum} ovaries.

A similar case appears to apply to *bcd* transcription, which was found to be specifically controlled by Serendipity-δ (Sry-δ), a zinc finger protein. *sry*-δ null alleles block oogenesis, and only a particular genetic combination revealed the specific requirement of *sry*-δ for *bcd* transcription (PAYRE *et al.* 1994). Thus, both *psq* and *sry*-δ have a basic function in oogenesis, probably through the transcriptional control of other germline genes, and a specific function in the control of *tor* and *bcd*, respectively. This similarity is particularly intriguing considering the peculiarities of early Drosophila embryogenesis and the fact that anterior patterning by *bcd* seems to be restricted to Diptera (CASCI 2000) and that *tor*-dependent terminal patterning appears in Diptera and Coleoptera, but not in Hymenoptera (WILSON and DEARDEN 2009). Thus, the regulation of *bcd* and *tor* transcription by a specific function of more general germline transcription factors might be related to their particular recruitment to embryonic patterning. Interestingly, in the case of terminal patterning, *tor* transcription appears to be regulated independently from that of genes encoding the other elements of the signaling pathway (*e.g.*, Ras and Raf), as induced expression of *tor* is sufficient to rescue the *psq*^{rum} terminal phenotype. However, the only essential function of these other elements of the Tor pathway in the oocyte is to transmit the Tor signal, as indicated by the phenotype of mutant germline clones (AMBROSIO *et al.* 1989). Thus, in the absence of *tor* activity, these products appear to be silent in the Drosophila germline. Altogether, these data suggest a possible multiple-step way to acquire new regulatory mechanisms in a given set of cells. This possibility appears particularly suggestive in the light of recent results pointing to Tor as the receptor for prothoracicotropic hormone (PTTH), which stimulates the production of the molting hormone ecdysone (REWITZ *et al.* 2009). Could this be a more ancient role of *tor* that would subsequently have been recruited for embryonic terminal patterning in some insects? Such a scenario appears to apply to the Toll signaling pathway, which shares some similarities with the Tor pathway (CASANOVA *et al.* 1995), and has a widely conserved function in immunity in many animals (LEULIER and LEMAITRE 2008) and whose components are also transcribed by the Drosophila female germline to specify the embryonic dorsoventral pattern.

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GENETICS

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Control of Germline *torso* Expression by the BTB/POZ Domain Protein Pipsqueak Is Required for Embryonic Terminal Patterning in Drosophila

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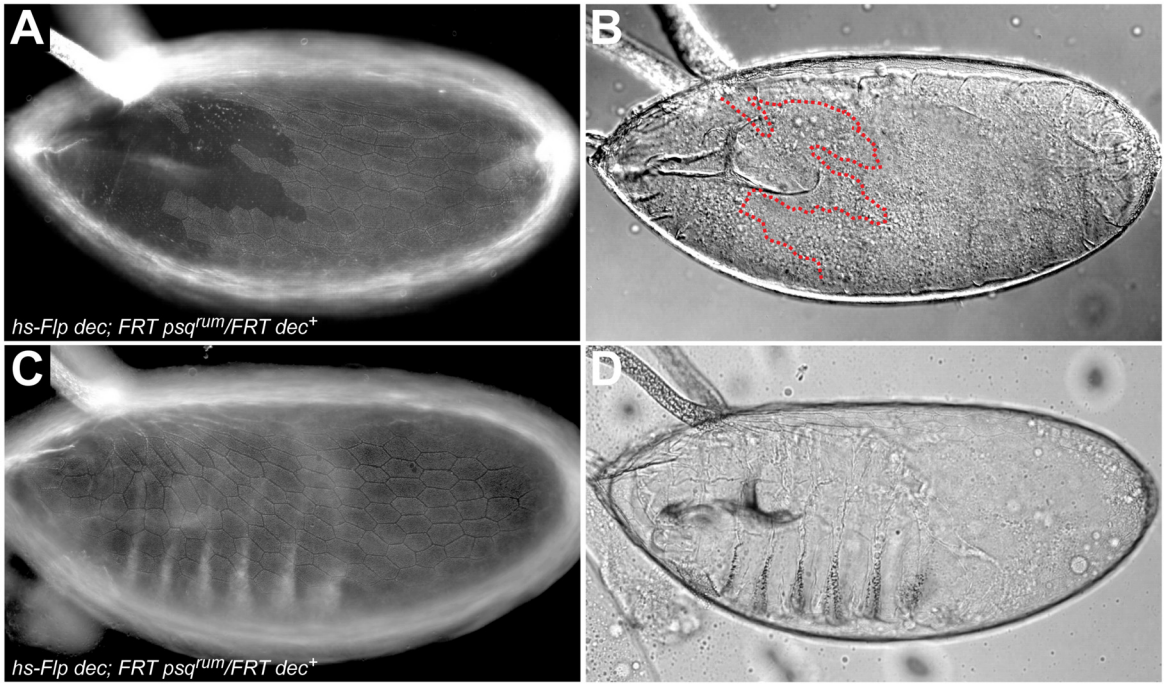

FIGURE S1.—*psq*^{rum} mutant follicle cell clones do not affect terminal patterning. (A,B) *psq*^{rum} somatic clones in the follicle epithelium do not affect terminal regions in the embryo. *psq*^{rum} mutant follicle cells are marked by the loss of the *dec*$^{+}$ marker, resulting in a transparent chorion. A large *dec*$^{-}$; *psq*^{rum} clone covers the anterior part of the egg shown in (A). Note the presence of a normal head skeleton in the phase contrast view shown in (B). The border of the clone is indicated by a hatched red line in (B). (C,D) Conversely, *psq*^{rum} germline clones produce terminal phenotypes even when somatic follicle cells are wild-type as detected by the absence of *dec*$^{-}$ clones. (A) and (C) are dark-field images. (B) and (D) are phase-contrast images of the embryos shown in (A) and (C), respectively.

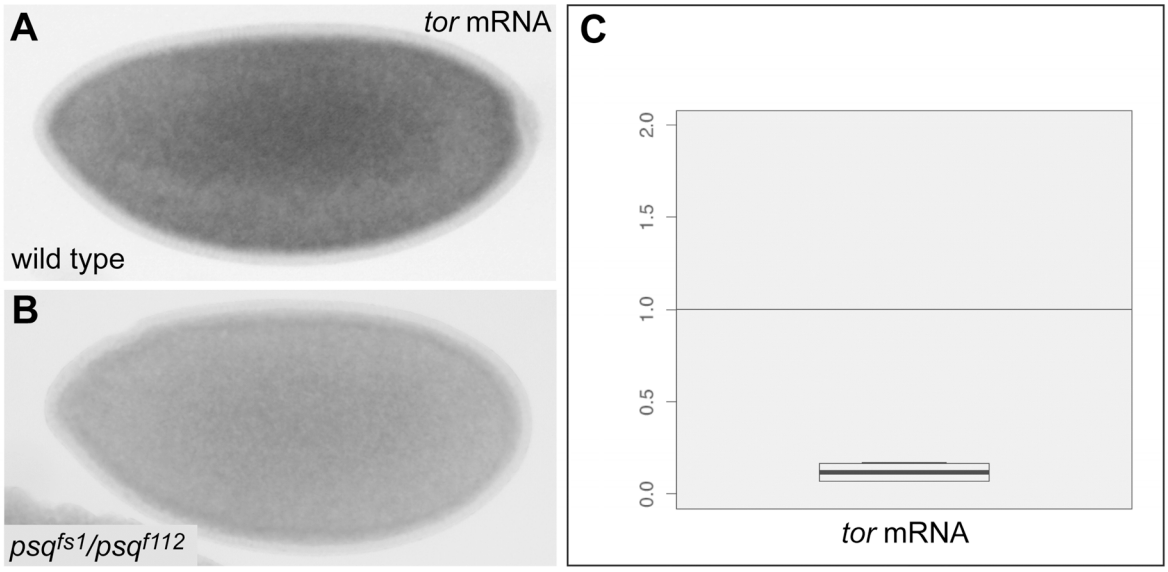

FIGURE S2.—*tor* mRNA levels are reduced in ovaries and embryos from *psq*^{fs1}/*psq*^{f112} transheterozygous females (A,B) *tor in situ* hybridization in wild-type embryo (A) and *psq*^{fs1}/*psq*^{f112} mutant (B). *tor* mRNA levels are strongly reduced in *psq*^{fs1}/*psq*^{f112} embryos. (C) *tor* mRNA is eight-fold reduced in *psq*^{fs1}/*psq*^{f112} ovaries compared to the wild type, as detected by real-time PCR. The Y-axis indicates relative transcript levels as fold-change between wild-type and *psq*^{fs1}/*psq*^{f112} ovaries.

TABLE S1

Analysis of heteroallelic combinations between *psq*^{rum} and other *psq* alleles or deficiencies uncovering *psq*

psq alleles or deficiencies	homozygous phenotype	maternal-effect phenotype of heteroallelic combination with *psq*^{rum}	nature of mutation	reference
psq[rum]	All embryos show terminal defects (lack of structures posterior to A7; n=100). In addition embryos frequently show deletions of one or more abdominal segments.		EMS-induced; G to A nucleotide substitution affecting the splicing donor site of intron 6, which is common to all known *psq* transcripts. This leads to partial retention of the intron in the mRNA and is predicted to cause the addition of 16 amino acids followed by a stop codon.	this work
psq[8109]	Decreased viability of adults, female sterile, posterior group, dorsalized eggshell and embryo, early oogenesis defects.	96% of embryos show little cuticle, 4% of embryos develop contiguous cuticle and lack terminal structures (n=100)	P{PZ} insertion into the largest intron of *psq-1*. Aberrant fusion protein created.	HOROWITZ and BERG 1995
psq[0115]	Decreased viability of adults, female sterile, posterior group, dorsalized eggshell and embryo, early oogenesis defects.	94% of embryos show little or no cuticle, 6% of embryos develop contiguous cuticle and lack terminal structures (n=100)	P{PZ} insertion into the largest intron of *psq-1*. Aberrant fusion protein created.	HOROWITZ and BERG 1995
psq[fs1]	Female sterile, posterior group, grandchildless	Embryos do not hatch. 80% of embryos lack segment A8 and/or spiracles (n=50)	P{lacW} insertion into the first intron of *psq-1*.	SIEGEL *et al.* 1993
psq[rev2]	n.d. *	Fertile	Isolated as EMS-induced revertant of *psq* overexpression in the eye. Deletion of 11 bp, resulting in a premature stop codon in the BTB domain. The P{GSV1}lola[GS88A8] insertion is still present on the chromosome.	FERRES-MARCO *et al.* 2006

psq[rev4]	n.d. *	Many collapsed eggs. 6.25% of embryos develop contiguous cuticle. These embryos lack abdominal segments (n=80).	Isolated as EMS-induced revertant of *psq* overexpression in the eye. Nucelotide substitution causing a Q530>stop mutation. The P{GSV1}lola[GS88A8] insertion is still present on the chromosome.	FERRES-MARCO *et al.* 2006
psq[rev7]	n.d. *	Fertile	Isolated as EMS-induced revertant of *psq* overexpression in the eye. Amino acid replacement M1I in the start Methionine of Psq1. The P{GSV1}lola[GS88A8] insertion is still present on the chromosome.	FERRES-MARCO *et al.* 2006
psq[rev9]	n.d. *	Weakly fertile. Adults show narrow blistered wings with extra veins. Embryos do not show terminal or other morphological defects (n=50).	Isolated as EMS-induced revertant of *psq* overexpression in the eye. G to A nucleotide substitution affecting the splicing donor site of intron 2 (72 bp), leading to retention of the intron in the mRNA. This causes the in-frame addition of 24 amino acids within the BTB/POZ domain, presumably disrupting the conserved BTB pocket domain, which is important for dimerization. The P{GSV1}lola[GS88A8] insertion is still present on the chromosome.	FERRES-MARCO *et al.* 2006
psq[rev12]	n.d. *	90% of embryos show little or no cuticle, 10% of embryos develop contiguous cuticle and lack terminal structures (n=80)	Isolated as EMS-induced revertant of *psq* overexpression in the eye. Amino acid replacement G867D in the third PSQ repeat. The P{GSV1}lola[GS88A8] insertion is still present on the chromosome.	FERRES-MARCO *et al.* 2006

psq[rev14]	n.d. *	98% of embryos show little or no cuticle, 2% of embryos develop contiguous cuticle and show posterior and terminal defects (n=100)	Isolated as EMS-induced revertant of *psq* overexpression in the eye. Nucleotide substitution causing a Q582>stop mutation. The P{GSV1}lola[GS88A8] insertion is still present on the chromosome.	FERRES-MARCO *et al.* 2006
psq[KG02404]	Viable, female-sterile	Embryos do not hatch. 60% of embryos lack segment A8 and/or spiracles (n=50)	P{SUPor-P} insertion in *psq-1* 5'-UTR	BELLEN *et al.* 2004
psq[KG00811]	Lethal	Fertile	P{SUPor-P} insertion in the largest intron of *psq-1*, 0.7 kb downstream of the transcription start site of *psq-3* transcripts	BELLEN *et al.* 2004
psq[EP2011]	Viable, female-sterile. Homozygous females lay no eggs.	Embryos do not develop cuticle (n=100).	P{EP} insertion in the largest intron of *psq-1*, 71 bp upstream of the transcription start site of *psq-3* transcripts	BELLEN *et al.* 2004
Df(2R)47A		Most embryos undeveloped, few embryos with little cuticle		FlyBase
Df(2R)E3363		Most embryos undeveloped, few embryos develop and show terminal defects		FlyBase

Phenotypes of embryos from females carrying heteroallelic combinations of psq^{rum} are indicated. *: We were unable to determine homozygous phenotypes of the *psq* revertant alleles, all of which are lethal in homozygous state and in all trans-heterozygous combinations tested. Lethality of these chromosomes is presumably due to the presence of additional mutations or due to the P{GSV1}lola[GS88A8] P-element insertion.

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