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Functional characterization of the role of Imp, a *Drosophila* mRNA binding protein, during oogenesis

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Functional characterization of the role of Imp, a *Drosophila* mRNA binding protein, during oogenesis

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Dedication

To my parents

for their love and support

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Establishment of cell polarity requires the involvement of several posttranscriptional regulatory mechanisms, including mRNA localization and translational control. A family of highly conserved RNA binding proteins in vertebrates, VICKZ (Vg1RBP/Vera, IMP-1, 2, 3, CRD-BP, KOC, ZBP-1) proteins, has been shown to act in these two processes. Previous studies of the posttranscriptional mechanisms mediated by VICKZ family members have been largely limited by the lack of genetic approaches in certain vertebrate systems. Identification of Imp, the *Drosophila* member of the VICKZ family, opened the possibility to use genetic approaches to investigate the roles of a VICKZ family member in mRNA localization and translational control.

In this dissertation, we show that Imp is associated with Squid and Hrp48, two heterogeneous proteins (hnRNP) that complex with one another to regulate localized expression of *gurken (grk)*. In addition, Imp binds *grk* mRNA with high affinity in vitro

and is concentrated at the site of *grk* localization in midstage oocytes. Mutation of the *Imp* gene does not substantially alter *grk* expression, but does partially suppress the *grk* mis-expression phenotype of *fs(1)k10* mutants. In contrast, overexpression of Imp in germ line cells results in mislocalization of *grk* mRNA and protein. The opposing effects of reduced and elevated Imp activities on *grk* expression suggest that *Imp* acts in regulation of *grk* expression, but in a redundant way.

To further explore the mechanisms by which localized expression of *grk* is regulated by Imp, a deficiency screen was conducted to search for dominant modifiers of the dorsalized phenotype resulting from Imp overexpression. Twelve genomic regions were identified to contain dominant modifiers of the Imp overexpression phenotype. Further characterization of mutants of genes within these genomic regions led to identification of five modifiers, including *cyclin E (cycE)*, *E2f transcriptional factor 1 (E2f1), lingerer (lig), snail (sna)* and *mushroom body expressed (mub)*. *E2f1* encodes a transcriptional factor that is involved in regulating the G1 to S phase transition during mitosis. Mutation of *E2f1* results in altered *grk* mRNA and protein distribution within oocyte, revealing a role for this gene in regulation of *grk* expression.

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Chapter One: General introduction

OVERVIEW OF mRNA LOCALIZATION AND TRANSLATIONAL CONTROL

Establishment of cell polarity is a fundamental process underlying formation of higher order multicellular structures in a variety of organisms. One strategy to achieve cellular asymmetry is through unequal distribution of cytoplasmic determinants within an individual cell (Betschinger and Knoblich, 2004). The restricted distribution of those cytoplamic determinants can be accomplished by co-ordination of two post-transcriptional mechanisms, mRNA localization and translational control (reviewed by Lipshitz and Smibert, 2000; Palacios and St. Johnston, 2001; Kindler et al., 2005).

mRNA localization is a widely employed mechanism to produce high levels of protein products at their site of function. It is considered more energy efficient than deploying proteins themselves to their target site, since one mRNA molecule can serve as a template for multiple rounds of protein synthesis, and most localized mRNAs are usually associated with components of the translation machinery (Jansen, 2001). The mechanisms of mRNA localization have been studied extensively in recent years. Localization signals, or cis-acting elements, have been identified in various localized transcripts. They generally reside in the 3' untranslated regions (3'UTR) of mRNAs and are recognized by trans-acting factors, or mRNA binding proteins, to direct transport of mRNA cargo to specified regions. Proper localization of mRNA also requires an intact cytoskeleton and involves actin- or microtubule-based motor proteins which travel along the cytoskeleton (reviewed by Jansen, 2001; Kindler et al., 2005).

mRNA localization is usually coupled with translational control to ensure precise control of the expression of cytoplasmic determinants. During the process of transporting mRNAs to their destination site, translation of these mRNAs is generally repressed to avoid ectopic expression, which could otherwise result in severe developmental defects (Kwon et al., 1999; Lipshitz and Smibert, 2000). Upon arrival at the destination site, translation of these transcripts is activated. In addition to its role during mRNA transport, translational regulation is also required to silence the unlocalized transcripts, especially for some mRNAs that are inefficiently localized. One striking example comes from the study of *nanos* mRNA localization in *Drosophila. nanos* transcripts localize to the posterior of oocyte and early embryo where locally expressed Nanos protein direct posterior body patterning (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991; Gavis and Lehmann, 1992). The *nanos* mRNA localization process is very ineffective, since only 4% of the total *nanos* transcripts are localized at the posterior of the embryo (Bergsten and Gavis, 1999). Translation of the unlocalized *nanos* transcripts is blocked, whereas translation of the localized transcripts is active. Translational repression of *nanos* mRNA is mediated by a 90 nucleotide translational control elements (TCE) on *nanos* 3'UTR, which is recognized by Smaug, a RNA binding protein (Dahanukar and Wharton, 1996; Dahanukar et al., 1999; Smibert et al., 1999; Gavis et al., 1996).

Drosophila oogenesis and embryogenesis are excellent systems to study posttranscriptional mechanism. Axis determination in oocytes and early embryos relies on restricted distribution of maternal cell determinants, which are under tight control

through a combination of mRNA localization and translational control mechanisms. Large scale genetic screens for mutants with defective body patterning have identified localized body patterning determinants, as well as trans-acting factors that are required for localization of these determinants (reviewed by St Johnston and Nusslein-Volhard, 1992). Among all the identified localized maternal transcripts, *bicoid (bcd), nanos (nos),* and *oskar (osk)* are essential for establishment of the anteroposterior body axis, while *grk* is responsible for formation of the dorsoventral axis as well as the anteroposterior axis (reviewed by van Eeden and St Johnston, 1999; St Johnston and Nusslein-Volhard, 1992; Nilson and Schupbach, 1999). Mechanisms of how they are localized have been studied extensively though a combination of biochemical, cellular and genetic approaches. Recent research progress will be described below on the posttranscriptional control mechanism of *bcd, osk,* and *grk* localization during oogenesis or early embryogenesis, with a special focus on *grk* mRNA localization and translational control.

POSTTRANSCRIPTIONAL CONTROL MECHANISMS OF *bcd* AND *osk*

Anterior-posterior body plan in *Drosophila* early embryo relies on proper deployment of three maternal body determinants, Bcd, Osk and Nos to their specific regions (van Eeden and St Johnston, 1999).

bcd encodes an anterior body patterning morphogen, which forms an anterior to posterior protein gradient to specify head and thorax structure in *Drosophila* embryo (Frohnhofer et al., 1986; Driever and Nusslein-Volhard, 1988a; Driever and Nusslein-

Volhard, 1988b). Pre-localization of *bcd* mRNA to the anterior pole of oocyte and egg is essential to achieve the Bcd protein gradient (Frigerio et al., 1986; Berleth et al., 1988). Following transcription in the interconnected nurse cells, *bcd* mRNA is transported into the oocyte and is concentrated at the anterior margin of the oocyte where it persists until early embryonic stage (Berleth et al., 1988; St Johnston et al., 1989). Upon egg activation, translation of localized *bcd* transcripts is activated through cytoplasmic polyadenylation (Salles et al., 1994). The anterior localization of *bcd* mRNA is mediated through over 600 nt localization signal in the *bcd* 3'UTR (Macdonald and Struhl, 1988). Within the 600 nt localization signal, a minimal region, the IV/V RNA (stem-loops IV and V of the whole localization signal), has been shown to be both necessary and sufficient to direct the early phase of *bcd* mRNA localization during oogenesis (Macdonald and Kerr, 1997). Three trans-acting factors, *exuperantia (exu*), *swallow (swa),* and *staufen (stau*) are required for *bcd* mRNA localization at different stages (Berleth et al., 1988; Stephenson et al., 1988; St Johnston et al., 1989). In ovaries of *exu* mutants, localization of *bcd* mRNA is altered during early stages and results in a evenly distribution of the *bcd* transcripts in later stage oocyte. However, in *swa* and *stau* mutant ovaries, *bcd* mRNA localization is affected only at late stages of oogenesis (Berleth et al., 1988; Stephenson et al., 1988; St Johnston et al., 1989).

Osk, another maternal body determinant, is deposited at the posterior pole of oocytes to nucleate assembly of the pole plasm, a cytoplasmic region which contains the abdominal and germline determinants (Ephrussi et al., 1991; Ephrussi and Lehmann,

1992; Kim-Ha et al., 1991). Restriction of Osk protein to the posterior pole is critical, since ectopic expression of Osk elsewhere will result in anterior patterning defects and formation of extra germ cells (Smith et al., 1992; Ephrussi and Lehmann, 1992). Posterior accumulation of Osk protein is achieved though three processes: pre-localization of *osk* mRNA, translational repression prior to mRNA localization and Osk protein mediated anchoring. Similar to *bcd* mRNA, *osk* transcripts are synthesized in nurse cells and then transferred to oocyte (Kim-Ha et al., 1991; Ephrussi et al., 1991). During early oogenesis, *osk* mRNA is enriched in oocyte. As the oocyte expands rapidly at stage 7-8, a transient accumulation of *osk* mRNA is observed at the anterior margin of the oocyte, followed by local concentration of *osk* mRNA at the posterior pole, where it remains until embryogenesis (Kim-Ha et al., 1991; Webster et al., 1994; Rongo et al., 1995). The localization signal directing posterior concentration of *osk* mRNA resides in the *osk* 3'UTR (Webster et al., 1994). Unlike *bcd,* localization signal of *osk* mRNA is more complicated. A systematic deletion analysis has led to identification of several elements within the *osk* 3'UTR that are essential for different steps in the localization process: transport of *osk* transcripts from nurse cell to oocyte, transient accumulation at the anterior margin of oocyte and final localization to the posterior pole (Kim-Ha et al., 1993). Nevertheless, properties of these elements still remain uncharacterized.

The posterior localization of *osk* mRNA is microtubule (MT) dependent, since the localization process is disrupted by microtubule depolymerizing drugs as well as mutations in genes such as *grk*- and *par 1*- that alter polarity of the microtubule network

(Clark et al., 1994; Gonzalez-Reyes et al., 1995; Martin and St Johnston, 2003; Roth et al., 1995; Shulman et al., 2000; Theurkauf, 1994). A plus end directed motor protein, Kinesin, has been shown to co-localize with *osk* mRNA at the posterior pole in mid-stage oocytes (Clark et al., 1994). In addition, *osk* mRNA fails to localize properly to the posterior pole in *kinesin heavy chain* mutants, which suggests that Kinesin couples *osk* mRNA cargo to the MT network and moves it towards the plus end of MT (Brendza et al., 2000; Duncan and Warrior, 2002).

A number of trans-acting factors required for posterior localization of *osk* mRNA have been identified though maternal screens. Mutations of these genes disrupt the *osk* mRNA localization process without affecting the cytoskeleton organization. One of those trans-factors, *staufen (stau),* encodes a double stranded RNA binding protein, which co-localizes with *osk* mRNA at the posterior pole of the oocyte (St Johnston et al., 1991; St Johnston et al., 1992). In *stau* mutants, *osk* mRNA remains at the anterior margin of the oocyte from stage 9, and fails to localize to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). However, it is still unclear whether Stau specifically binds to the cis-acting elements in the *osk* 3'UTR.

In contrast to the early appearance of *osk* transcripts in the oocyte, Osk protein is only detected at the posterior pole of the oocyte from stage 8 onwards when *osk* mRNA starts to concentrate there (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). This is achieved by translational repression of *osk* mRNA prior to localization and translational activation of the *osk* transcripts upon arrival at the posterior pole.

Translational repression of *osk* mRNA is mediated by a RNA binding protein, Bruno, which binds to several conserved sequence on *osk* 3'UTR, the Bruno Responding Elements (BREs). Mutations in the BREs result in disruption of Bruno binding to *osk* mRNA as well as premature Osk protein accumulation in early stage oocyte (Kim-Ha et al., 1995; Webster et al., 1997).

Unlike most localized mRNAs, maintenance of *osk* transcripts at the posterior pole of the oocyte requires Osk protein activity. In *osk* nonsense mutants which produce truncated Osk protein (protein terminated at the N-terminal half), *osk* transcripts fail to localize properly at the posterior pole of the oocyte after midoogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). In contrast, the twelve known *osk* missense mutations do not lead to any *osk* mRNA localization defect (Ephrussi et al., 1991; Kim-Ha et al., 1991). Therefore, Osk protein is essential for anchoring *osk* transcripts at the posterior pole. A positive feedback model for *osk* mRNA localization is proposed. After *osk* mRNA is deposited into the oocyte, it is translationally inactive. Upon arrival at the posterior pole of the oocyte, *osk* transcripts are actively translated into protein, which in turn, stabilizes and maintains the *osk* mRNA at the posterior pole (Markussen et al., 1995; Rongo et al., 1995; Rongo et al., 1997; Vanzo and Ephrussi, 2002).

POST-TRANSCRIPTIONAL CONTROL MECHANISMS OF *grk*

grk is involved in both AP and DV axis determination

Establishment of anterior-posterior (AP) and dorsal-ventral (DV) polarity in *Drosophila* oocytes relies on a series of communication events between the oocyte and the surrounding somatic follicle cells, which are mediated by Grk, a transforming growth factor-α-like protein (Schupbach, 1987). Grk is initially expressed in the oocyte and later secreted to the intercellular space between oocyte and follicle cells (Neuman-Silberberg and Schupbach, 1993, 1996), where it binds to Torpedo (Top), an EGFR (Epidermal Growth Factor Receptor) receptor expressed on all follicle cell membranes and triggers downstream *Egfr* signaling pathway (Kammermeyer and Wadsworth, 1987; Sapir et al., 1998).

The AP and DV axis of egg and embryo are specified sequentially by two rounds of *grk/Egfr* signaling events taking place at different time during oogenesis. In each case, *Egfr* signaling is activated in a unique subpopulation of follicle cells, which can be accomplished by spatially restricted distribution of *grk* transcripts within the oocyte. Prior to stage 7, *grk* mRNA is localized at the posterior of the oocyte, where it is actively translated and signals to the neighboring follicle cells to adopt posterior follicle cell fates (Neuman-Silberberg and Schupbach, 1993; Gonzalez-Reyes et al., 1995; Roth et al., 1995). Those cells, in turn, send an unidentified signal back to the oocyte, which leads to reorganization of the microtubule network. As a result, *bcd* and *osk mRNA* are localized to the anterior margin and the posterior pole of the oocyte, respectively, which defines the polarity along the AP axis (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In addition, the oocyte nucleus migrates from the posterior to the anterior cortex. *grk* mRNA, which

closely associates with the oocyte nucleus, also moves to the anterior region of the oocyte and forms a transient anterior ring. By stage 9, the *grk* transcripts are concentrated at the anterodorsal corner and form a crescent above the oocyte nucleus. The spatially restricted Grk protein activates *Egfr* signaling in the adjacent follicle cells and establishes dorsal follicle cell fates (for review, see Nilson and Schupbach, 1999). In strong *grk* mutants or mutants with dramatically reduced *grk-Egfr* signaling, both AP and DV polarity are disrupted, resulting in ventralized eggshells which have no or fused dorsal appendages, as well as a duplication of the micropyle, an anterior chorion structure that becomes duplicated at the posterior end of the eggshell (Gonzáles-Reyes et al., 1995; Roth et al., 1995).

Mechanisms involved in *grk* mRNA localization

Cis-acting elements required for *grk* mRNA localization

Proper localization of *grk* mRNA within the oocyte is mediated by cis-acting elements on *grk* transcripts. Unlike most localized mRNAs identified so far, whose localization signals reside exclusively in the 3'UTR (Reviewed by Palacios and St. Johnston, 2001), a 64 nucleotide region in the 5' coding sequence of *grk* mRNA, referred to as GLS (*grk* localization signal), appears to be both necessary and sufficient for *grk* mRNA localization at the anterodorsal corner within the oocyte, according to an *in vivo* injection assay with fluorescently labeled *grk* RNA (Thio et al., 2000; Van De Bor et al., 2005). This 64 nucleotide region is highly conserved across *Drosophila* species and predicted by mfold program to form a stem loop structure (Van De Bor et al., 2005). The

mRNA of *I* factor, a non-LTR retrotransposon in *Drosophila*, shares the consensus stem loop structure and localizes to same region as *grk* transcripts, implicating that *grk* and *I* factor might compete for the same localization machinery (Van De Bor et al., 2005).

Source of *grk* transcripts

The close association of *grk* mRNA with the oocyte nucleus has led to the hypothesis that *grk* mRNA is produced primarily or exclusively in the oocyte nucleus, which facilitates the localization of *grk* mRNA to the apical side of the nucleus (Goodrich et al., 2004; Norvell et al., 1999; Palacios and St. Johnston, 2001; Saunders and Cohen, 1999). In this model, the microtubule network is not required for transporting *grk* mRNA from nurse cell into oocyte. This hypothesis is supported by evidence from colchicine experiment, in which wild type flies were fed with colchicine, an inhibitor of microtubule polymerization, to block transport of all mRNA from nurse cells into the oocyte. In ovaries of colchicine treated flies, *grk* mRNA remains associated with oocyte nucleus at the anterodorsal corner, while *osk* mRNA, as a control, is enriched only in the nurse cells (Saunders and Cohen, 1999).

However, this model would require *grk* to be an exceptional transcript derived from oocyte nucleus, which is arrested in meiotic metaphase I and transcriptionally quiet during oogenesis (King and Burnett, 1959; Spradling, 1993). Instead, *grk* mRNA could be synthesized in nurse cells and transported into the oocyte, similar to *bcd* and *osk* transcripts (Johnstone and Lasko, 2001; Lipshitz and Smibert, 2000; Palacios and St. Johnston, 2001). To test this alternative possibility, a standard mitotic recombination

technique was employed to generate mosaic eggchambers in which *grk* can be produced only in the nurse cell, but not the oocyte (Caceres and Nilson, 2005). The nurse cell derived Grk localizes to the anterodorsal corner of the oocyte and polarizes both AP and DV axis correctly, suggesting that transcription of *grk* occurs in the nurse cells, and this is sufficient for establishment of both body axes (Caceres and Nilson, 2005). This experiment, however, does not exclude the possibility that the oocyte nucleus contributes some *grk* transcripts, which are not required for formation of AP and DV axis. Therefore, further experiments are still needed to address whether nurse cells are the only source for *grk* transcripts and, more importantly, to investigate the mechanism involved in transporting *grk* transcripts from nurse cells into the oocyte and its subsequent localization at the anterodorsal corner of the oocyte.

***grk* mRNA localization is dependent on microtubule network**

The asymmetric distribution of *grk* transcripts during oogenesis relies on a polarized microtubule (MT) network. During stage 2-6, the microtubule organization center (MTOC) is located diffusely at the posterior of the oocyte, from where the MTs extend their plus ends though the ring canals into the nurse cells (Clark et al., 1994,1997; Theurkauf et al., 1992). The polarized MTs thus direct localization of *grk* mRNA to the posterior of the oocyte. In *maelstrom (mael)* mutant, the MTOC is mislocalized to an anterior or lateral position before stage 7, as visualized by Nod:β-gal, a microtubule minus end marker and centrosomin, a centriole marker (Clark et al., 1997; Li and Kaufman, 1996; Clegg et al., 1997, 2001). As a result, *grk* mRNA is detected at the

lateral region, rather than its normal position at the posterior of the oocyte (Clegg et al., 1997, 2001).

During stage 7, posterior follicle cells send a signal with an unknown nature to the oocyte, which triggers a rearrangement of the MT network and the migration of the oocyte nucleus from the posterior to the anterior cortex (reviewed by Riechmann and Ephrussi, 2001). The posterior MTOC disintegrates and a new MTOC appears at the anterior margin of the oocyte, revealed by Nod:β-gal, centrosomin (Li and Kaufman, 1996), and the nucleating factor, γ-Tubulin at 37C (γTub37C) (Schnorrer et al., 2002). Injection of fluorescently labeled *grk* mRNA into live oocytes revealed a novel feature of the anterodorsal localization of *grk* mRNA during midoogenesis (MacDougall et al., 2003). The *grk* mRNA assembles into particles within the oocyte and moves in two steps: first toward the anterior cortex and then toward the oocyte nucleus at the dorsal region (MacDougall et al., 2003). Each of these steps requires MT and Dynein, a minus end MT motor, as the localization process of injected *grk* mRNA can be abolished by greatly reducing Dynein heavy chain activity, or by treatment with MT depolymerizing drugs, but not actin depolymerizing drugs (MacDougall et al., 2003). Based on the evidence from high resolution imaging of MTs within the oocyte, a hypothesis is proposed that there exist two perpendicular sets of MTs within the stage 7-9 oocyte, which might be responsible for each step of grk mRNA transport (MacDougall et al., 2003; Januschke et al., 2002, 2006). In the first step, *grk* mRNA is transported along the MT arrays which emanate from the anterior cortex and extend their plus ends to the posterior pole of the oocyte (MacDougall et al., 2003). For the second step, *grk* mRNA

travels along the MTs that are nucleated from the oocyte nucleus-centrosome complex at the anterodorsal corner and run parallel to the nurse cell-oocyte boundary (MacDougall et al., 2003; Januschke et al., 2002, 2006). In both steps, *grk* mRNA is directed toward the minus end of the MT, which is mediated by Dynein. Further experiments are still needed to elucidate the mechanisms underlying the formation of these two distinct MT arrays, and whether this two-step movement model can be applied to the nurse cell derived *grk* transcripts.

Transacting factors required for *grk* mRNA localization and translational control

Extensive genetic screens searching for maternal-effect mutants with defective dorsoventral patterning have identified a number of trans-acting factors that are essential for *grk* mRNA localization and translational control. Two classes of trans-acting factors have been well characterized and appear to regulate localized *grk* expression at different levels. One of these mutants, the spindle class mutants, produce eggs with a ventralized chorion, resembling the loss-of-function phenotype of *grk* (reviewed by Morris and Lehmann, 1999). While the other group of mutants, including *fsk10, sqd, hrp48* and *otu,* produce eggs with the opposite phenotype, dorsalized eggshells, indicating a *grk* gain-of-function phenotype (Wieschaus, 1979; Wieschaus et al., 1978; Serano et al., 1995; Norvell et al., 1999; Kelley, 1993a; Goodrich et al., 2004). Their roles in *grk* expression will be discussed in detail in the following session.

spindle genes

The spindle class mutants are a group of female sterile mutants that produce eggs with fused or no dorsal appendages, which result from a failure to accumulate Grk protein during midoogenesis (reviewed by Morris and Lehmann, 1999). In most spindle class mutants, including *spindle-A (spn-A), -B, -C, -D, -E* and *okra (okr),* the reduction of Grk protein levels is always preceded by a delay of *grk* mRNA localization within the oocyte: the *grk* transcripts form a ring around the anterior margin of the oocyte at stage 9 and become restricted to the anterodorsal region before stage 10b (González-Reyes et al., 1997). Thus the failure to accumulate Grk protein in these spindle mutants might be due to translational repression of the unlocalized *grk* mRNA. However, in other spindle mutants including *aubergine (aub)* and *encore (enc),* the *grk* mRNA mislocalization defects are either non-existent or very mild in contrast to the high penetrance of Grk protein accumulation defects, indicating that genes associated with these mutations might be involved in activation of *grk* mRNA translation directly (Wilson et al., 1996; Hawkins et al., 1997).

Cloning of the spindle genes has revealed an unexpected link between dorsoventral patterning and DNA repair. Among the characterized spindle class genes, *spn-A, -B, -C, -D* and *okr* are homologous to genes in the yeast RAD52 epistasis group, which function in the recombinational repair of double-stranded DNA breaks (DSB) (Ghabrial et al., 1998; Kooistra et al., 1997; Staeva-Vieira et al., 2003; Abdu et al., 2003). Besides the dorsoventral patterning defects, mutations in these *spn* genes also cause defects in meiotic progression, reflected by abnormal oocyte nuclear morphology, as well

as a reduction of meiotic recombination frequency and an increase of non-disjunction rate, indicating that the meiotic DSBs might be left unrepaired in these mutants (Ghabrial et al., 1998; Kooistra et al., 1997; Staeva-Vieira et al., 2003; Abdu et al., 2003). In yeast, the unrepaired DSBs activate an ATM/ATR-dependent cell check point in meiosis and mitosis, which leads to an arrest of cell cycle progression to allow enough time for DNA repair (Roeder and Bailis, 2000). Ghabrial et al thus hypothesize that an analogous meiotic check point in response to the presence of unrepaired DSBs is also activated in these *spn* mutants, which results in meiotic progression defects and a failure to accumulate Grk protein (Ghabrial and Schupbach, 1999). According to this model, if the DSBs are not produced in the first place, the meiotic check point will not be activated; therefore, meiotic progression and Grk accumulation will not be affected in these *spn* mutants. In agreement with this prediction, the mutant phenotypes of *spn B-D* and *okr* are suppressed by mutations in *mei-W68*, the *Drosophila* homologue of *spo 11*, which is essential for DSB formation in yeast (McKim and Hayashi-Hagihara, 1998; Roeder, 1997; Ghabrial and Schupbach, 1999; Staeva-Vieira et al., 2003; Abdu et al., 2003). In addition, based on the hypothesis, if the meiosis check point is eliminated, then the meiotic progression and Grk accumulation will appear normal in these *spn* mutants, regardless of the presence of unrepaired DSBs. As expected, mutations in *mei-41*, a *Drosophila* member of the ATM/ATR subfamily of phosphatidylinositol-3-OH-kinase-like protein, also suppress the patterning and meiotic defects in these *spn* mutants, indicating that block of Grk accumulation in these spindle mutants depends on a *mei-41* mediated check point (Ghabrial and Schupbach, 1999; Abdu et al., 2002). An eIF4a–like

translation initiation factor, Vasa, has been implicated as an downstream target of this check point to regulate Grk translation, as the spindle phenotype and meiotic defects of *vasa* mutant are not suppressed by *mei-41* or *mei-W68* mutations, and Vasa protein appears to be posttranslationally modified in *spn* mutants (Ghabrial and Schupbach, 1999). However, it is still not clear how the unrepaired DSBs lead to activation of the *mei-41* dependent check point, as well as how the activated check point result in posttranslational modification of Vasa.

k10. sqd, hrp48* and *otu

In contrast to the ventralized phenotype produced by *spindle* mutants, *sqd*, *k10, hrp48* and *otu* mutants produce eggs with the opposite phenotype, dorsalized eggshells, a gain-of-function phenotype arising from mislocalization of *grk* mRNA to the entire anterior cortex, instead of restriction to the anterodorsal corner of the oocyte (Wieschaus et al., 1978; Kelley, 1993b; Goodrich et al., 2004; Neuman-Silberberg and Schupbach, 1993). Unlike most mutants with defective *grk* mRNA localization, the unlocalized *grk* message is actively translated, suggesting a failure to repress translation of unlocalized *grk* mRNA in *sqd, hrp48, k10* and *otu* mutants. The resulting Grk protein expansion along the DV axis induces excess dorsal follicle cell fates, which leads to dorsalization of the egg and future embryo (Wieschaus et al., 1978; Kelley, 1993b; Goodrich et al., 2004; Neuman-Silberberg and Schupbach, 1993).

Sqd is a member of heterogeneous nuclear ribonucleoproteins that are involved in various cellular processes such as splicing, transcription, nuclear transport, mRNA

localization, translational control and protein stability (reviewed by Dreyfuss et al., 1993; Krecic and Swanson, 1999; Dreyfuss et al., 2002). Alternative splicing of *sqd* generates three isoforms, SqdA, SqdS and SqdB (Norvell et al., 1999). They have different subcellular distribution and perform distinct roles in the localized expression of *grk.* SqdA, which is predominantly cytoplasmic, facilitates efficient translation of *grk* mRNA. In contrast, SqdS is enriched in germline nuclei and required in nuclear export and cytoplamic localization of *grk* mRNA within the oocyte. Proper localized expression of grk requires both isoforms. The third isoform, SqdB, is present in germline nuclei and does not appear to play a role in *grk* mRNA localization or translational control (Norvell et al., 1999).

GST-pull down assay implicates that Sqd interacts physically with K10, a putative transcriptional factor (Norvell et al., 1999). In *fs(1)k10* mutant, Sqd accumulation is lost in the oocyte nucleus, indicating that K10 might regulate the accumulation of Sqd in the oocyte nucleus (Norvell et al., 1999). It still remains unclear how k10 mediates regulation of *grk* mRNA localization mechanistically.

The dorsalized phenotype of eggs produced by *sqd* mutant females can be enhanced by mutations in *hrp48* and *otu*, which encode a heterogeneous nuclear ribonucleoprotein and a RNA binding protein involved in mRNA localization, respectively (Goodrich et al., 2004). Therefore, *sqd, hrp48* and *otu* might co-operate to regulate *grk* expression. This idea is further supported by evidence from immunoprecipitation experiments, which revealed that these three proteins interact with each other in a RNA dependent manner (Goodrich et al., 2004). In addition, Sqd and

Hrp48 have been shown to bind the *grk* 3'UTR specifically by UV cross-linking analysis, indicating that they have a more direct role in regulation of *grk* expression (Goodrich et al., 2004). These results have lead to a model that Sqd and Hrp48 associate with *grk* transcripts in the nucleus to facilitate its export to the cytoplasm, where the RNP is joined by Otu and other unidentified partners, to localize *grk* mRNA to the anterodorsal corner of the oocyte (Goodrich et al., 2004)

In addition to their roles in *grk* localization, *sqd* and *hrp48* are also involved in *osk* mRNA localization and translational control. The supporting evidence comes from the following experiments. First, Sqd and Hrp48 colocalize with *osk* mRNA at the posterior pole of the oocyte during midoogenesis (Norvell et al., 2005; Huynh et al., 2004; Yano et al., 2004). Second, in *sqd* and *hrp48* mutants, *osk* mRNA is mislocalized to the middle of the oocyte, which might be an indirect result of altered microtubule networks (Steinhauer and Kalderon, 2005; Yano et al., 2004). Third, the mislocalized *osk* mRNA is actively translated, indicating that *osk* mRNA localization and translational control is uncoupled in *sqd* and *hrp48* mutants (Steinhauer and Kalderon, 2005; Yano et al., 2004; Huynh et al., 2004). The presence of shared components in regulation of both *grk* and *osk* mRNA localization indicates that the regulatory mechanisms of these two mRNAs are very similar. It will be interesting to identify other shared components, as well as distinct factors, which confer specificity to each regulatory complex.

VICKZ FAMILY PROTEINS

VICKZ proteins involve in mRNA localization and translational control

Intracelluar mRNA localization and controlled translation require the recognition of cis-acting RNA elements by trans-acting RNA binding proteins. A family of highly conserved RNA binding proteins in vertebrates, VICKZ (Vg1RBP/Vera, IMP-1, 2, 3, CRD-BP, KOC, ZBP-1) proteins have been suggested to function in these two processes (Deshler et al., 1997; Deshler et al., 1998; Ross et al., 1997; Havin et al., 1998; Doyle et al., 1998; Mueller-Pillasch et al., 1997).

One founding member of the VICKZ family proteins, Vg1RBP (also known as Vera), is associated with ER and is involved in localization of Vg1 mRNA to the vegetal cortex of early *Xenopus* embryos (Deshler et al., 1998; Havin et al., 1998; Zhang et al., 1999; Kwon et al., 2002). Vg1 mRNA encodes a transforming growth factor-β protein that is involved in mesoderm formation and left-right axis determination. Localization of the Vg1 mRNA to the vegetal cortex is medicated by a 360-nt region in the Vg1 3'UTR: the vegetal localization element (VLE) (Mowry and Melton, 1992). Vg1RBP binds with high affinity to repeated UUCAC motif in the VLE (Deshler et al., 1997). Deletion of the UUCAC motifs results in a decrease of both Vg1RBP binding and Vg1 mRNA localization, which indicates that binding to the UUCAC motifs within the VLE is essential for proper Vg1 mRNA localization (Deshler et al., 1997).

Another family member, Zip code Binding Protein1 (ZBP1), has been implicated in post-transcriptional control of *β-actin* mRNA, which is localized at the leading edge of chicken embryo fibroblasts (CEF) and the growth cone of developing neurons (Ross

et al., 1997; Bassell et al., 1998; Lawrence and Singer, 1986). A conserved 54-nucleotide localization signal known as the "zipcode" within the 3'UTR of *β-actin* mRNA is both necessary and sufficient for *β-actin* mRNA localization (Kislauskis et al., 1993). ZBP1 binds to the zip code with high affinity and co-localizes with *β-actin* mRNA to the protrusions of CEFs and developing neurons (Ross et al., 1997; Oleynikov and Singer, 2003; Zhang et al., 2001). In addition, expression of ZBP1 in a *ZBP1* deficient cell line induces *β-actin* mRNA localization (Oleynikov and Singer, 2003), which supports the idea that ZBP1 mediates the localization of *β-actin* mRNA to the leading edge of CEFs and the growth cones of neurons. During the journey from nucleus to the leading edge of CEFs, *β-actin* mRNA is translationally repressed en route to avoid ectopic expression, until it reaches its destination. Translational repression of β-actin transcripts is also mediated by ZBP1 in a zip code dependent manner. ZBP1 prevents the translation of *β-actin* transcripts by inhibiting the joining of 40S and 60S subunits of the ribosome, which is essential for translation initiation (Huttelmaier et al., 2005). Upon arrival at the periphery of the cell, ZBP1 is phosphorylated by a locally expressed tyrosine kinase Src, which results in dissociation of ZBP1 from the *β-actin* mRNA. Therefore, translational repression of *β-actin* mRNA is abolished (Huttelmaier et al., 2005).

The VICKZ family also includes the mouse *c-myc* coding region determinant binding protein, CRD-BP, which is involved in regulating mRNA stability (Doyle et al., 1998), and three human insulin-like growth factor II mRNA binding proteins (Imp1-3) indicated in translational repression of IGF-II mRNAs, as deletion of the IGF-II mRNA binding sites for IMPs leads to defective translation of the mutant transcripts (Nielsen et

al., 1999). Members of this family are often overexpressed in various tumors, suggesting a role in carcinogenesis (Yaniv and Yisraeli, 2002).

Imp is a *Drosophila* homologue of VICKZ proteins

Members of the VICKZ family are very similar at structural level (Yaniv and Yisraeli, 2002). They are composed almost entirely of RNA binding domains. The vertebrate members contain two NH_2-terminal RRM (RNA recognition motif) and four KH (hnRNP-K homology) domains, while the *Drosophila* and *C.elegans* homologues lack the two RRM domains. Structural analysis of Vg1RBP/Vera and ZBP1 reveals that the KH domains, not the two RRM domains, bind to their RNA targets specifically (Git and Standart, 2002). Therefore, the KH domains of VICKZ family members might be responsible for recognition of mRNA targets and play a conserved role in mRNA localization and translational control.

The *Drosophila* member of the VICKZ family, Imp, is identified by Nielsen et al (Nielsen et al., 2000). Unlike its vertebrate homologues, Imp lacks the two RRM domains. The amino acid identity of Imp with any other VICKZ family member is around 47% (Nielsen et al., 2000). However, the individual KH domain of Imp shows much higher homology to its counterparts in ZBP1 than to each other, indicating that Imp is a member of this family. The structural similarity between Imp and other VICKZ family members strongly suggests that Imp might be also involved in mRNA localization and translational control in *Drosophila*.

Previous studies of the posttranscriptional control mechanisms mediated by VICKZ family members have been largely limited by the lack of genetic approaches in vertebrate systems. For example, to determine whether Vg1RBP is required for localization of Vg1 mRNA, antibody against Vg1RBP is injected to the *Xenopus* oocyte, which leads to defective localization of the Vg1 mRNA (Kwon et al., 2002). However, partial localization of the Vg1 mRNA is still observed after treatment, which either means that other RNA binding proteins might play redundant roles in the localization process, or the activity of Vg1RBP is not completely eliminated (Kwon et al., 2002). Therefore, it is not certain that Vg1RBP is essential for Vg1 mRNA localization. One way to resolve this problem is to examine Vg1 mRNA localization in a *vg1RBP* mutant background, which is an unrealistic approach in *Xenopus* oocytes. Identification of *Imp* has opened the possibility to use genetic approaches to study function of a Vicky family member.

The initial description of Imp by Nielsen et al indicates that Imp mRNA displays biphasic expression during embryogenesis: with an even distribution of maternally derived Imp transcripts in early stage embryos, and concentration of zygotically transcribed Imp mRNA in the central nervous system of later stage embryos (Nielsen et al., 2000).

OVERVIEW OF THE DISSERTATION

In this thesis, a combination of genetic and biochemical approaches were employed to characterize the function of *Drosophila Imp*. Here we show that Imp is associated with Squid and Hrp48, which have been shown to form a RNP complex to regulate localized expression of *grk* (Goodrich et al., 2004). In addition, Imp colocalizes with *grk* and *osk* mRNAs in midstage oocytes. Mislocalization of *grk* mRNA also causes an alteration of Imp distribution. Overexpression of Imp altered the expression pattern of *grk* and *osk*, with no effect on *bcd* mRNA. In contrast, loss-of-function of *Imp* mutations have no obvious phenotypes during oogenesis, but it does suppress the dorsalized phenotype caused by fs(1)K10 mutants. The opposing effects of reduced and elevated *Imp* activity on *grk* mRNA expression indicates a role of *Imp* in *grk* mRNA regulation. The physical interaction with the *grk* 5'UTR and grk 5'CDS further strengthens the idea that Imp forms a RNP complex with Hrp48 and Sqd to regulate *grk* expression.

To further explore the role of Imp in *grk* expression, a modifier deficiency screen was designed to search for dominant modifiers of the dorsalized phenotype in a sensitized Imp overexpression background. We have screened 421 deficiency stocks from Exelixis Inc., which together cover about 56% of the whole *Drosophila* genome and identified 12 modifiers. Among the identified modifiers, 4 have been matched to individual genes, which includes *cyclin E (cyc E)*, *E2f transcriptional factor 1 (E2f1), lingerer (lig), snail (sna)* and *mushroom body expressed (mub)*. Both *E2f1* and *cyc E* are involved in the G1 to S phase transition during mitosis (reviewed by Vidwans and Su, 2001). Mutation in

E2f1 results in altered *grk* mRNA and protein distribution within oocyte, thus suggesting a role in regulation of *grk* expression.

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Chapter Two: Imp associates with Squid and Hrp48 and contributes to localized expression of *gurken* in the oocyte

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Running Title: Regulation of *grk* mRNA by Imp

ABSTRACT

Localization and translational control of Drosophila *gurken* and *oskar* mRNAs rely on the hnRNP proteins Squid and Hrp48, which are complexed with one another in the ovary. Imp, the Drosophila homolog of proteins acting in localization of mRNAs in other species, is also associated with Squid and Hrp48. Notably, Imp is concentrated at sites of *gurken* and *oskar* mRNA localization in the oocyte, and alteration of *gurken* localization also alters Imp distribution. Imp binds *gurken* mRNA with high affinity in vitro; thus, the colocalization with *gurken* mRNA in vivo is likely to be the result of direct binding. *Imp* mutants support apparently normal regulation of *gurken* and *oskar* mRNAs. However, loss of *Imp* activity partially suppresses a *gurken* misexpression phenotype, indicating that *Imp* does act in control of *gurken* expression, but has a largely redundant role that is only revealed when normal *gurken* expression is perturbed. Overexpression of Imp disrupts localization of *gurken* mRNA, as well as localization and translational regulation of *oskar* mRNA. The opposing effects of reduced and elevated *Imp* activity on *gurken* mRNA expression indicate a role in *gurken* mRNA regulation.

INTRODUCTION

The restriction of proteins to discrete subcellular regions can be accomplished by a coordinated program of mRNA localization and translational control. These mechanisms are used prominently during oogenesis in Drosophila, where several localized proteins direct body patterning. The dorsoventral axis of the oocyte and later the embryo are established by a process that involves the specific expression of Gurken (Grk) protein at a dorsal position near the anterior of the oocyte. Similarly, patterning along the anteroposterior axis relies on restricted expression of Bicoid (Bcd) and Oskar (Osk) proteins at the anterior and posterior poles, respectively, of the oocyte and embryo. In each case, the deployment of the protein is a consequence of localization of the mRNA to the appropriate region within the oocyte, coupled with translational controls to enhance accumulation of the protein at this destination (reviewed by Johnstone and Lasko, 2001).

These programs of post-transcriptional control of gene expression require RNA binding proteins that recognize regulatory elements within the mRNAs and mediate association with the localization or translational control machinery. Although it has proven difficult in most cases to demonstrate that a particular protein/RNA interaction contributes to regulation, multiple RNA binding proteins are required for correct expression of *grk* mRNA; these include Squid (Hrp40), Hrp48 (also known as Hrb27C), Bruno (Bru), Vasa, and Otu (Kelley, 1993; Styhler et al., 1998; Tomancak et al., 1998; Norvell et al., 1999; Filardo and Ephrussi, 2003; Goodrich et al., 2004; Yan and

Macdonald, 2004). Each of these proteins is also required for correct expression of *osk* (Tirronen et al., 1995; Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995; Webster et al., 1997; Huynh et al., 2004; Yano et al., 2004; Steinhauer and Kalderon, 2005; Norvell et al., 2005), revealing substantial similarities in the control of *grk* and *osk* mRNAs.

Mutants defective for Sqd, Hrp48 and Otu have a common *grk* mRNA localization defect (Goodrich et al., 2004). Normally, *grk* mRNA is transiently localized to the anterior of the oocyte at stage 8 of oogenesis, and then becomes restricted to the dorsal side of the anterior. In the mutants, *grk* mRNA persists along the anterior and fails to localize dorsally. Because localization of *grk* mRNA has been suggested to result from two vectorial movements - one towards the anterior, and a second directed dorsally (MacDougall et al., 2003)- these genes could act specifically in the second movement. Sqd and Hrp48 have also been implicated in translational regulation, and act to limit the translation of *grk* mRNA to the fraction of the mRNA that is properly localized at the dorsal side of the oocyte (Norvell et al., 1999; Goodrich et al., 2004). The mechanistic details of how these proteins contribute to localization and translational control remain to be determined, but it does appear that they function as part of a regulatory RNP complex, since Hrp48 interacts physically with both Sqd and Otu (Goodrich et al., 2004). Two components of the complex, Sqd and Hrp48, have been suggested to assemble with the mRNAs in the nucleus, and associate with other factors in the cytoplasm (Matunis et al., 1992a; Matunis et al., 1992b; Matunis et al., 1993; Norvell et al., 1999). It is likely that additional members of this complex have not yet been identified.

One candidate for another regulatory factor is Imp, the Drosophila homolog of a family of proteins that act in post-transcriptional regulation in a variety of animals (Ross et al., 1997; Yaniv and Yisraeli, 2002). One of the founding members of the family, ZBP-1, binds to a localization element in the chicken beta-actin mRNA (Kislauskis et al., 1994), and appears to direct localization to the leading edge of embryonic fibroblasts (Farina et al., 2003). Another founding member, the Xenopus Vg1RBP/VERA protein, binds to signals directing localization of Vg1 and VegT mRNAs to the vegetal pole of the oocyte (Deshler et al., 1997; Deshler et al., 1998; Havin et al., 1998; Kwon et al., 2002). Mammalian homologs, the Imp proteins, have been suggested to act in mRNA localization (Runge et al., 2000), mRNA stability (Doyle et al., 1998) and translational regulation (Nielsen et al., 1999). A recent report examined the RNA binding properties of Drosophila Imp protein, focusing specifically on the *osk* mRNA and its possible regulation by Imp (Munro et al., 2006). Although mutation of candidate Imp binding sites in the *osk* mRNA did block accumulation of Osk protein, loss of imp activity did not cause a similar defect.

Here we also characterize the Drosophila Imp protein, and show that it interacts with Sqd and Hrp48, two proteins that regulate expression of *osk* and *grk* mRNAs. Mutation of the *Imp* gene does not substantially alter *grk* or *osk* expression. Nevertheless, the *Imp* mutant partially suppresses a *grk* mis-expression phenotype, arguing that it does contribute to *grk* regulation, but may act redundantly and does not have an essential role. Consistent with this interpretation, overexpression of Imp interferes with localization of *grk* mRNA.

RESULTS

Imp associates with hnRNP proteins Sqd and Hrp48

Co-immunoprecipitation experiments with ovary extracts were performed to test for association of Imp with proteins known to act in post-transcriptional regulation. We find that Imp co-immunoprecipitates with Sqd and with Hrp48 (Fig 2.1). Both Sqd and Hrp48 are, like Imp, RNA binding proteins, and their association with Imp could involve only protein/protein contacts, or could depend on RNA binding. The co-immunoprecipitations were also performed after treatment with RNase, and in each case the interaction is disrupted. An additional RNA binding protein, Nanos, was also tested by the same assay, but did not co-immunoprecitate with Imp. Thus, the RNA-dependent association of Imp with Sqd and Hrp48 is specific, and is not a common property of all RNA binding proteins.

Imp is concentrated at the site of *grk* mRNA localization

Imp protein is cytoplasmic and present in essentially all cells of the ovary, both somatic follicle cells and the germ line nurse cells and oocyte (Fig 2.8). Within the germline, Imp displays a changing pattern of abundance in different cells. At the earliest stages of oogenesis Imp is initially uniform in the dividing germline cells of each cyst, but becomes rapidly concentrated in the oocyte (Fig 2.8). This enrichment is lost by stage 7, after which the level of Imp in the oocyte is noticeably reduced. Although the uniform level of Imp in the oocyte decreases, Imp levels become elevated in during stages 8 and 9

in the narrow zone between the nucleus and the anterior and dorsolateral margins of the oocyte (Fig 2.2A). This is precisely the region in which *grk* mRNA and protein accumulate, with Grk protein then trafficking to the follicle cells to provide a localized signal in the pathway that specifies dorsal fates (Nilson and Schupbach, 1999). The enrichment of Imp could be specific, and perhaps related to the localization or translation of *grk* mRNA. Alternatively, all cytoplasmic proteins might display a concentration in this restricted region of the ooplasm. To distinguish between these options we examined other cytoplasmic proteins that also appear in the oocyte. Unlike Imp, neither Vasa (Fig 2.2 D, E) nor Spindle E (data not shown) proteins were enriched between the nucleus and the oocyte margins, despite being present throughout the ooplasm. Thus the regional concentration of Imp is specific.

To determine if the concentration of Imp correlates with localization of *grk* mRNA, we examined Imp protein distribution under conditions when the anterodorsal localization of *grk* mRNA is altered (Fig 2.3). In *sqd* mutant ovaries *grk* mRNA remains concentrated at the anterior of the oocyte, but is no longer restricted to the dorsal region (Norvell et al., 1999). The concentration of Imp at the dorsal side of the nucleus is substantially reduced in the mutant ovaries, and the degree of residual localization correlates well with the level of residual localized Grk protein expression. Furthermore, Imp still appears concentrated along the anterior of the oocyte, just as does *grk* mRNA. To quantitate the loss of dorsal localization, anti-Imp fluorescence intensity levels were measured in the dorsal cortical region and in a more posterior cortical region for multiple wild type and *sqd* mutant oocytes (see Experimental Methods). The dorsal/posterior ratio

was 1.54-2.00 for wild type, reflecting the dorsal concentration, and 1.07-1.24 for the *sqd* mutant, confirming that dorsal localization of Imp is reduced (Fig 2.3). Thus, Imp not only colocalizes with *grk* mRNA, but may rely on the *grk* mRNA localization machinery or *grk* mRNA itself for that distribution. Consequently, Imp could bind directly to *grk* mRNA and act in regulation of its expression.

Imp protein also colocalizes with *osk* mRNA at the posterior pole of the oocyte (Fig 2.2B), raising the possibility that it is associated with *osk* mRNA. Munro et al. (Munro et al., 2006) examined this association in detail, and found that Imp remained colocalized with *osk* mRNA that had been mispositioned in various mutants, very strongly arguing that Imp is bound, directly or indirectly, to *osk* mRNA.

Imp binds to *grk* mRNA

The RNA binding activity of purified Imp was monitored using a quantitative nitrocellulose filter binding assay. RNA probes for the assay were prepared from different parts of the *grk* and *osk* mRNAs (Fig 2.4C), as well as from *bcd* mRNA 3' UTR, which serves as a negative control (there is no indication of any role for Imp in *bcd* expression). Imp binds with highest affinity to the *grk* mRNA 5' UTR (K_d is 134 nM) and 5' part of the coding sequence (K_d of 192 nM)(Fig. 4A and D). Other parts of the *grk* mRNA, including the 3' coding region and 3' UTR, bind with much lower affinity (K_d of 1.6 and 4.3 μM, respectively). Weak binding is also observed for the *osk* 3' UTR (various segments bind with a K_d of 0.77-3.4 μM)(Fig 2.4B and 2.4D). The negative control RNA

is largely unbound at similar Imp protein concentrations. The high affinity binding of Imp to *grk* mRNA, taken together with the colocalization of the mRNA and protein in the oocyte, strongly suggests that Imp binds *grk* mRNA in vivo. It also appears possible that Imp binds directly to *osk* mRNA in vivo. However, the lower affinity of the in vitro interaction with *osk* mRNA raises the possibility that binding occurs in the context of an RNP complex in which multiple proteins contact the mRNA and a high affinity is acheived through multiple contacts, much as has been suggested for the binding of a localization complex to the *bcd* mRNA (Arn et al., 2003). Obvious candidates for additional complex components are the Imp-associated proteins Sqd and Hrp48, both of which are concentrated with Imp and *osk* mRNA at the posterior pole of the oocyte (Yano et al., 2004; Huynh et al., 2004; Norvell et al., 2005).

Imp mutants have no overt ovarian phenotype, but suppress the dorsalization of *fs(1)K10*

For genetic analysis of *Imp* function we used a P element insertion mutant, Imp^{G0072}, in which the transposon is inserted into the *Imp* gene. Imp^{G0072} is semi-lethal, with rare escapers surviving as adults for up to several days. Two forms of Imp protein are detected in wild type ovaries by western blot analysis (Fig 2.1). The most abundant form is about 70 kDa, consistent with the reported structure of the Imp protein. Both protein forms are present at greatly reduced levels in Imp^{G0072}/ *Df(1)H133* females. The Imp^{G0072} chromosome was extensively backcrossed to wild type to

remove secondary mutations. Reversion of the mutation by excision of the P element restored full viability and expression of Imp protein.

Homozygous Imp^{G0072} females, although unhealthy and prone to getting stuck in the growth medium, can produce eggs before they die. The eggs appear phenotypically normal and, if fertilized by wild type sperm, form viable and fertile adults. Not surprisingly then, the *Imp* mutants have no substantial defects in distribution or activity of localized mRNAs that contribute to embryonic body patterning (data not shown). Nevertheless, *Imp* could play a redundant role, perhaps in regulation of *grk* or *osk* given its concentration at the sites where these mRNAs are localized and its association with Hrp48 and Sqd.

As a more sensitive assay for a role in the dorsoventral pathway we asked if the *Imp* mutation could modify an existing dorsoventral patterning defect. Mutation of *fs(1)k10*, which encodes a transcriptional factor, results in mislocalization of *grk* mRNA and protein along the anterior margin of the oocyte during midoogenesis, instead of restriction to the anterodorsal corner. Consequently, eggs laid by mutant *fs(1)K10* mothers are strongly dorsalized and display expansion and fusion of the two dorsal appendages that normally lie near the dorsal midline (Wieschaus et al., 1978; Roth and Schupbach, 1994; Serano et al., 1995; Neuman-Silberberg and Schüpbach, 1996) (Fig 2.5D). When *fs(1)k10* females are also homozygous for the Imp^{G0072} mutation, the dorsalization phenotype is partially suppressed (Fig 2.5C-D). In keeping with the absence of a detectable Imp^{G0072} mutant phenotype, the *fs(1)K10* eggshell phenotype is only fully suppressed in very rare cases. More commonly, the embryos from the *Imp fs(1)K10*

mothers display some degree of reduced dorsalization, as scored by the effects on the dorsal appendages. No substantial change in the *grk* mRNA distribution of the *fs(1)K10* mutant accompanies the partial suppression of the eggshell phenotype (data not shown). This is not surprising, given the normal appearance of *grk* mRNA distribution in *Imp* mutants. However, because the eggshell phenotype provides a very sensitive measure of *grk* patterning activity, it can presumably reveal defects not discernable by the in situ hybridization assay. We conclude that reduction of *Imp* activity has a very weak effect on dorsoventral patterning.

Overexpression of Imp alters dorsovental polarity and expression of *grk*

As an alternate assay for Imp activity in dorsoventral patterning we overexpressed the protein in the germline cells of the ovary using the GAL4/UAS expression system (Brand and Perrimon, 1993; Rorth, 1998). Overexpression of Imp produces a strong and highly penetrant effect: dorsalization of the eggshell. In wild type, embryos have an eggshell in which the two anterior dorsal appendages lie close to the dorsal midline, and are separated from one another (Fig 2.6E). Only 17% of the embyros from Imp overexpression mothers (n=255) have the wild type dorsal appendages. The remainder of the embryos show various degrees of dorsalization, in which the dorsal appendages fuse and form an anterior ring around the embryo (Fig 2.6 F-H). The Imp overexpression also causes a partial dumpless phenotype (a defect in transfer of nurse cell contents to the ooycte), such that the embryos are smaller than normal (data not shown).

Consistent with the eggshell dorsalization phenotype, *grk* mRNA and protein fail

to be tightly restricted to the anterodorsal corner of the oocyte, and are instead dispersed along the anterior margin. At stage 9, 97% of Imp overexpression oocytes display an abnormal circular ring of *grk* mRNA (Fig 2.6B), with only 3% having the normal anterodorsal restriction (n=31)(Fig 2.6A). By stage 10 localization has improved, with 52% showing the ring and the remainder appear wild type (n=33). For Grk protein 79% of stage 9 oocytes have an anterior ring of Grk (Fig 2.6D), with 21% displaying the normal wild type anterodorsal distribution (n=53)(Fig 2.6C). Much as for the *grk* mRNA, the localization improves by stage 10, with 56% having an anterior ring and 44% wild type (n=45). Wild type controls show normal anterodorsal localization of both *grk* mRNA and protein at both stages. To address the possibility that the *grk* mRNA localization defect is an indirect consequence of altered microtubule organization, microtubules were monitored by imaging TauGFP in live egg chambers (Micklem et al., 1997). No substantial differences were observed in comparison of wild type and Imp overexpression oocytes (Fig 2.6 E and F, respectively). Similar results were obtained when microtubules were imaged by immunodetection in fixed samples (data not shown).

In wild type oocytes, *grk* mRNA is initially spread across the anterior of the oocyte, and becomes tightly restricted to the dorsal anterior before stage 9. Because localization improves in the Imp overexpression oocytes as development of the oocyte advances, it is possible that the actions that restrict the mRNA dorsally are active, but less effective or retarded. Alternatively, there may be mechanistically distinct phases in localization - one acting earlier and one later - with overexpression of Imp primarily or exclusively disrupting the early phase. Localization during the later phase would

gradually restore the wild type distribution of *grk* mRNA and protein.

Imp overexpression disrupts oocyte polarity and expression of *osk*

osk mRNA is tightly localized to the posterior pole of wild type oocytes from stage 9 thoughout the remainder of oogenesis, and Osk protein only accumulates after localization of the mRNA (Kim-Ha et al., 1995; Rongo et al., 1995; Markussen et al., 1995). In Imp overexpression ovaries *osk* mRNA [as well as Stau protein, which typically marks the distribution of *osk* mRNA; (Martin and St Johnston, 2003)] appears at two positions within individual oocytes: some of the *osk* mRNA is localized in a crescent at the posterior pole, the normal site of localization; and some of the *osk* mRNA appears in a discrete body in the ooplasm (Fig. 7D and G). These bodies, which are never seen in wild type (Fig 2.7C and E), are present in 79% of stage 9 egg chambers (n=113) and 55% of stage 10 egg chambers (n=99). Osk protein is present at both sites of *osk* mRNA concentration (Fig 2.7H), demonstrating that translation of *osk* mRNA is not negatively affected by Imp overexpression. Moreover, the accumulation of Osk is no longer dependent on posterior localization of the mRNA, revealing a loss of the regulation that normally prevents accumulation of Osk from unlocalized mRNA.

Imp is concentrated at the site of *osk* mRNA localization, and is associated with *osk* mRNA (Munro et al., 2006). Thus the *osk* mRNA localization defect arising from overexpression of Imp may involve a direct effect on *osk* mRNA. However, another consequence of Imp overexpression suggests that osk mRNA mislocalization may result, at least in part, from a more primary defect in microtubule organization. In wild type

stage 9 oocytes the microtubule polarity marker Kin-β-gal (Clark et al., 1994) is concentrated at the posterior pole (Fig 2.7I). When Imp is overexpressed, posterior localization of Kin-β-gal is greatly reduced (Fig 2.7L). However, the effects on microtubule organization must be subtle, as there are no obvious differences in microtubules imaging in wild type and Imp overexpression oocytes (Fig 2.6E, F), and *bcd* mRNA remains normally localized at the anterior of Imp overexpression oocytes (Fig 2.7B).

DISCUSSION

Deployment of proteins that control patterning in the oocyte relies on coordinated programs of mRNA localization and translational control. Many RNA binding proteins contribute to these programs, and some interact with one another in regulatory RNPs. Here we have shown that Imp is associated in an RNA-dependent manner with Sqd and Hrp48, and is thus part of a complex whose other members have clearly established roles in control of *grk* and *osk* expression. Imp does not have an essential role in regulation of either *grk* or *osk* mRNAs, as both mRNAs are expressed with no obvious defects in *Imp* mutant ovaries. However, loss of *Imp* activity does partially suppress the *grk* misexpression defect in *fs(1)K10* mutant oocytes, providing strong evidence that *Imp* contributes to regulation of *grk*. This view is reinforced by the colocalization of Imp with *grk* mRNA in vivo. Imp's role must be largely redundant, only becoming detectable when *grk* expression is perturbed. Overexpression of Imp has a much more dramatic effect, transiently blocking the dorsal localization of *grk* mRNA and disrupting localization and translational control of *osk* mRNA.

The evidence that Imp, Sqd and Hrp48 interact physically is complemented by striking similarities in *grk* and *osk* expression defects that arise from loss of *sqd* or *Hrp48* activity or from overexpression of Imp. In each case *grk* mRNA accumulates at the anterior of the oocyte, fails to become dorsally localized, and leads to misexpression of Grk protein. The defects of *sqd* and *Hrp48* mutants in *osk* expression may result from both direct and indirect effects: a direct effect via binding to *osk* mRNA, and an indirect effect owing to alterations in microtubule organization (Huynh et al., 2004; Yano et al.,

2004; Norvell et al., 2005; Munro et al., 2006). The same is true for Imp overexpression. In Imp overexpression oocytes the posterior localization of Kin-β-gal is disrupted, indicating some degree of microtubule defects. In addition, Imp, like Sqd and Hrp48, colocalizes with *osk* mRNA to the posterior pole of the oocyte.

The correlations between the consequences of excess *Imp* activity and loss of *sqd* or *Hrp48* activity may be significant, and suggest that Imp competes with these proteins at some level, either for binding to a common substrate or by exerting opposing effects on such a substrate. Alternatively, Imp could inactivate Sqd or Hrp48. Imp overexpression does not substantially alter the amount of Sqd or Hrp48 (data not shown), ruling out one form of inactivation. In addition, *sqd* and *Hrp48* mutants display one phenotype - altered polytenization of nurse cell nuclei (Goodrich et al., 2004)-that does not occur when Imp is overexpressed, arguing against any simple model in which Sqd and Hrp48 are inhibited by Imp.

Sqd and Hrp48 could compete with Imp at the level of RNA binding: excess Imp would displace Sqd or Hrp48 from shared or closely positioned binding sites on regulated mRNAs, yielding the same phenotype as if Sqd or Hrp48 were eliminated by mutation and thus not available for binding. This model seems unlikely, since Imp binds best to the 5' UTR and 5' coding regions of *grk* mRNA (regions implicated in *grk* mRNA localization; Thio et al., 2000; Van De Bor et al., 2005), while Sqd and Hrp48 bind to the 3' UTR (Norvell et al., 1999; Goodrich et al., 2004) (*osk* mRNA is considered below). However, Imp does bind with lower affinity to the *grk* mRNA 3' UTR, and the assays with Sqd and Hrp48 have not tested for binding to the *grk* mRNA 5' UTR.

Competition could also occur for events that transpire after RNA binding, with bound Imp promoting one outcome for the mRNA and bound Sqd and Hrp48 promoting another. For example, localization of *grk* mRNA has been suggested to involve two vectorial movements within the oocyte, one directed anteriorly and one directly dorsally (MacDougall et al., 2003). In this model Imp could promote the anterior movement, while Sqd and Hrp48 could contribute to the dorsal movement (a role in keeping with known phenotypes). Increasing the number of copies of one protein that become bound to the mRNA, even without a reduction in the binding of other proteins, could enhance association with the machinery that drives one vectorial movement, and thus alter the balance between the two movements. This type of interpretation would explain the partial suppression of the *fs(1)K10* ventralization phenotype by the *Imp* mutant. In the absence of K10 the competition would be skewed in favor of the Imp-promoted outcome. Removing Imp, even if it acts redundantly, could shift the competition back towards the balance normally achieved in wild type ovaries. This model might appear to be at odds with the known distributions of Imp, Hrp48 and Sqd. Specifically, Imp is colocalized with *grk* mRNA even after the proposed second vectorial movement of localization, while Hrp48 and Sqd are never detectably colocalized with the mRNA. However, the proposed competition would not require displacement of Imp from the mRNA, and Hrp48 and Sqd might act very early in the localization process (perhaps beginning in the nucleus where the proteins are concentrated) to orchestrate events that only occur later. Thus, the positions of the proteins in the ovary only rule out the possibility that they are

all persistently associated with one another, but do not argue against the models described here.

The defect in *grk* mRNA localization caused by overexpression of Imp is accompanied by ectopic accumulation of Grk protein, whose distribution mirrors that of *grk* mRNA along the anterior of the oocyte. In wild type ovaries *grk* mRNA is transiently concentrated along the anterior of the oocyte at stages 7 and 8, but there is no corresponding anterior ring of Grk protein (Saunders and Cohen, 1999). Thus the anterior accumulation of Grk when Imp is overexpressed reveals a defect in the control of *grk* mRNA translation, as well as localization. The premature translation could be an indirect consequence of derailing *grk* mRNA localization, or it could indicate a more direct effect of excess Imp on translation.

Does Imp act in regulation of *osk* mRNA?

Our discussion of *Imp* has focused on regulation of *grk* mRNA, since this role is supported by multiple lines of evidence. Overexpression of Imp also dramatically alters *osk* expression, acting indirectly by altering microtubule organization and perhaps acting directly through binding to *osk* mRNA. The data implicating Imp in *osk* regulation, whether direct or indirect, are substantially less compelling than for regulation of *grk*. Most importantly, we have no loss-of-function evidence that implicates Imp in *osk* mRNA regulation or in control of microtubule organization. Second, the binding of Imp to *osk* mRNA 3' UTR is relatively weak, with Kd values near or above 1 μM.

Munro et al (Munro et al., 2006) specifically explored the possible regulation of *osk* by Imp. They identified sequences (IBEs) in the *osk* mRNA as Imp binding sites. Inactivation of the IBEs eliminates accumulation of Osk protein. The *osk* mRNA initially localizes normally to the posterior of the oocyte, but is later delocalized and dispersed in the ooplasm, apparently an indirect consequence of a failure to accumulate Osk protein, which is required for anchoring of *osk* mRNA (Munro et al., 2006). Because loss of Imp activity did not cause similar defects, they concluded that another factor (factor X) must bind the IBEs for *osk* mRNA translation. Factor X could act redundantly with Imp, or factor X alone could mediate the action of the IBEs. Munro et al argue for the latter option, and propose a regulatory interplay between Imp and factor X, in which they compete for binding. By that model, overexpression of Imp would be expected to have consequences similar to mutation of the IBEs. The consequences of Imp overexpression differ when comparing our work to that of Munro et al. They present evidence that Imp overexpression reduces the level of Osk at stage 10, and do not report on *osk* mRNA localization. We also find a reduction in the accumulation of Osk protein at the posterior pole, but this is accompanied by mislocalization of a fraction of Osk protein to a discrete body in the ooplasm, a feature not observed for the IBE mutants. This body also contains *osk* mRNA, a localization defect that is clearly different from the dispersal of *osk* mRNA caused by mutation of the IBEs. To consider the possibility that the difference between our results and those of Munro et al. may reflect different levels of Imp overexpression, we varied the dosage of the *P[UAS-Imp]* transgene: increasing from one to two copies greatly enhanced the shift of *osk* mRNA and protein to multiple discrete bodies in the

ooplasm, but did not eliminate Osk accumulation (data not shown). Thus, in our extensive analysis the effects of Imp overexpression on *osk* mRNA localization and translation are markedly different from the IBE inactivation phenotype. We cannot explain why our results differ from those of Munro et al; further characterization of their Imp overexpression mutant might provide insights.

Imp mutants do not have an *osk* misexpression phenotype, but the in vitro binding properties of Imp and the consequences of Imp overexpression suggest that Imp plays a redundant role, much as we have argued in the case of *grk* mRNA. It would not be surprising for *Imp* to act in regulation of *osk*, as well as *grk*, since Imp is associated with proteins known to regulate both mRNAs.

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MATERIALS AND METHODS

Fly stocks and transgenics

A full length *Imp* cDNA (EST clone SD07045) was cloned into UASp vector (Rorth, 1998), and transgenic stocks were generated by standard methods. Multiple independent *P[UAS-Imp]* stocks produced similar phenotypes, with some differences in severity, when expressed from the *P[matα4-GAL4VP16] V37* driver.

Fly stocks *l(1)G0072* (now called Imp^{G0072}), *Df(1)HC133*, *P[matα4-GAL4VP16] V37* and $Dp(1;Y)v^{+}y^{+}$ were obtained from the Bloomington stock center. Secondary mutations on the w^{67c23} Imp^{G0072} chromosome were removed by extensive backcrossing to w^{1118} flies. The kinesin: LacZ reporter (Clark et al., 1994) was obtained from David Stein, the $fs(1)K10^{1}$ and sqd^{1} flies were from Trudi Schupbach, and the TauGFP flies were from Daniel St Johnston.

Plasmid rescue was performed to confirm that the P element of Imp^{G0072} is inserted into *Imp* gene. The lethality of Imp^{G0072} was confirmed to be due to the P element by isolation and characterization or revertants: five excision lines were obtained using the PΔ23 transposase, and none of them shows the lethality observed in Imp^{G0072}.

Homozygous Imp^{G0072} flies were obtained by the following cross scheme. $Df(1)v\text{-}L2/Dp(1;Y)v^{+}y^{+}$ males were crossed with $Imp^{G0072}/FM7c$ females. Progeny $Imp^{G0072}/\ Dp(1;Y)v^{+}y^{+}$ males were crossed with $Imp^{G0072}/FM7c$ females to get

Imp^{G0072} homozygous flies. Homozygous *fs(1)K10*^{1} *Imp*^{G0072} flies were obtained by the same strategy, using a *fs(1)K10*^{1} *Imp*^{G0072} chromosome obtained by recombination.

Generation of Imp antibody and purification of His-Imp

The coding region of the *Imp* gene was amplified by PCR and cloned into pET3b vector (Novagen). The Imp protein was expressed in E. coli Codon-Plus (Stratagene) and partially purified. Polyclonal antibody against Imp was raised by Josman LLC.

The *Imp* coding region was also cloned into PET15a vector (Novagen) to allow expression of Imp with an amino terminal 6xHis tag. The 6xHis-Imp fusion protein was expressed in E. coli Codon-Plus RP (Stratagene) and purified using Probond resin (Invitrogen).

Immunodetection and in situ hybridization

Ovaries were dissected and stained as described (Macdonald and Struhl, 1986; Macdonald et al., 1991). Primary antibodies were used at the following dilutions: rabbit anti-Imp, 1:600; rat anti-Vasa, 1:500; rabbit anti-Oskar, 1:4000; rat anti-Staufen, 1:100; rabbit anti-Stau, 1:1000; mouse anti-Gurken [1D12 from the Developmental Studies Hybridoma Bank (DSHB)], 1:10; mouse anti-beta-galactosidase (40-1a, DSHB), 1:40. Secondary antibodies were labeled with Cy5 (Jackson Immunoresearch Laboratories) or Alexa Fluor 488 (Molecular Probes). Stained ovaries were mounted in Vectashield medium(Vector Labs) and imaged with a Leica TCS-SP confocal microscope.

Live imaging of egg chambers was performed as described (Snee and Macdonald, 2004), using flies expressing TauGFP maternally to mark microtubules (Micklem et al., 1997).

To quantitate the loss of dorsal localization of Imp in *sqd* mutant oocytes, images acquired by confocal microscopy were analyzed for signal intensity using the ImageJ software (NIH). For each of four oocytes of each genotype, four non-overlapping boxes were drawn at random within the dorsal cortical region adjacent to the nucleus, or along the cortical region near the posterior pole. Signal intensity of each region was measured, to yield an average value, and the ratios of the dorsal and posterior values were determined. For the wild type oocytes the ratios were 1.54, 1.79, 1.83 and 2.00. For the *sqd* mutant oocytes the ratios were 1.07, 1.14, 1.14 and 1.24.

In situ hybridization was performed as described (Tautz and Pfeifle, 1989). Linearized plasmids containing the *osk* 3' UTR (pY107 cut by BamHI), the *bcd* 3' UTR (p908 cut by MluI) and the *grk* 3' UTR (p848 cut by BglII) were used as templates for synthesis of antisense RNA probes. The probes were labeled with Digoxigenin conjugated nucleotides (Roche Diagnostic GmbH).

Western blot analysis

Protein samples were electrophoresed in a 10% SDS-polyacrymide gel and electroblotted to PVDF membrane. Proteins were detected by chemiluminescence (Western Light, Tropix). Primary antibodies were affinity purified rabbit polyclonal anti-Imp at 1:3000, mouse monoclonal anti-α-Tubulin at 1:20,000 (gift from Tim Stearns),

mouse monoclonal anti-Sqd at 1:100 (gift from Trudi Schubach), and rabbit polyclonal anti-Hrp48 at 1:20,000 (gift from Don Rio).

Immunoprecipitation

Ovaries of w^{1118} flies were hand-dissected in PBS buffer, washed with lysis buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1mM EDTA, 1 mM DTT, 0.1% NP-40, 2 mM Pefabloc, 5 mM benzamidine. 2 μg/ml pepstatin, and 2 μg/ml leupeptin] three times, homogenized and cleared by centrifugation at 13,000 rpm for 25 minutes at 4°C. Aliquots of the extract (300 μl; equivalent to 50 ovary pairs) were incubated with primary antibody at 4°C for 1 hr. Subsequently, 20 μl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) pre-equilibrated with lysis buffer were added to the extract and incubated at 4°C for 30 minutes. Agarose beads were spun down and washed three times with lysis buffer. Next, they were incubated with lysis buffer with or without 50 ng/ml RNase A/T1 (Ambion) for 15 minutes at 4°C. Finally, beads were recovered with by centrifugation and washed with lysis buffer for three times. 2×SDS loading buffer (50 μl) was added to the beads and boiled at 100°C for 5 minutes. Samples were assayed by western blot.

Filter binding assay

Probes were generated by in vitro transcription in the presence of ^{32}P-UTP and gel purified. Details of the plasmids used to prepare the *osk* and *grk* RNAs described in

Fig. 4 are available on request. 20 μl reaction mix containing labeled probe (< 0.1 nM in final concentration) and various amounts of purified Imp protein in filter binding buffer [10 mM Tris-Cl (pH 8.0), 25 mM NaCl, 0.2 mM EDTA, 0.1mg/ml tRNA, 5 mg/ml heparin, 1mM DTT] were incubated on ice for one hour. Filter binding buffer (80 μl) was added to each reaction and the samples were filtered though nitrocellulose membrane filters (Millipore) pre-equilibrated with filter binding buffer at 4°C for at least one hour. The membrane filters were washed three times with 1 ml filter binding buffer and assayed for radioactivity by scintillation spectrometry. Dissociation constants (K_d) were calculated using Kaleidagraph (Synergy Software).

FIGURES

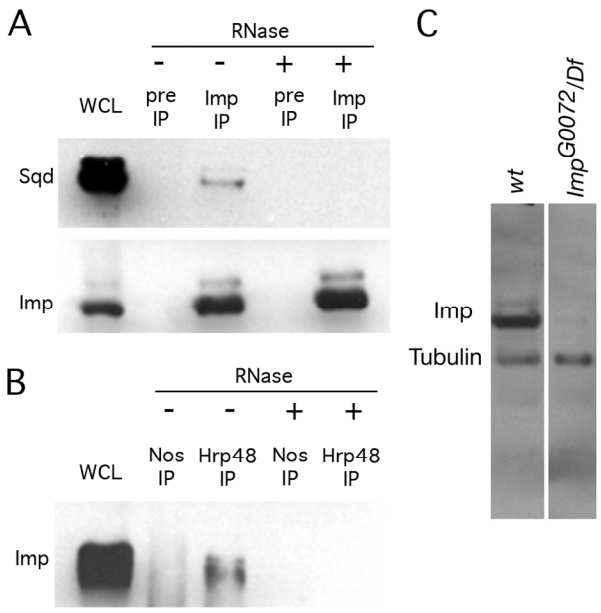


Figure 2.1 Imp is coimmunoprecipitated with Sqd and Hrp48.

A. Western blot of immunoprecipitation from ovary extract with α-Imp antibodies or preimmune serum (Pre), with or without RNase A/T1 treatment. Proteins on the blot were detected with α-Imp and α-Sqd. An amount of ovary extract equal to 5% of that used for the immunoprecipitations was loaded in lane WCL.

B. Western blot of immunoprecipitation from ovary extract with Hrp48 or Nanos antibodies, with or without RNase A/T1 treatment. The blot was probed with α-Imp.

C. Western blot analysis of Imp protein. Similar amounts of ovarian protein from w^{1118} or Imp^{G0072} */Df(1)HC133* mutant females were probed for Imp, and for alpha Tubulin as a loading control. The Imp^{G0072} hemizygous mutant has dramatically reduced levels of the two immunoreactive bands of Imp protein (one prominent and indicated as Imp, the other less abundant and slightly larger). The identities of the bands were confirmed in blots probed separately for Imp or Tubulin.

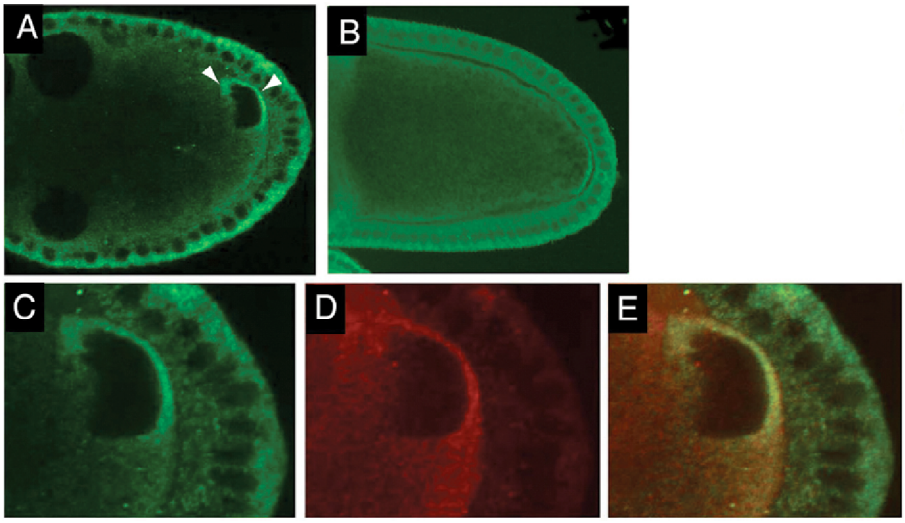


Figure 2.2 Distribution of Imp protein in the ovary.

A. Imp is transiently concentrated in an anterodorsal zone flanking the oocyte nucleus on the anterior and lateral sides (arrowheads). This localization can be detected as soon as the nucleus migrates to the anterior of the oocyte, and is largely lost by mid stage 9.

B. Imp is concentrated in a crescent at the posterior pole of the oocyte (arrowhead)

C-E. The anterodorsal concentration of Imp in the oocyte is specific. Egg chambers were double labeled for Imp (C) or Vas (D). Both proteins can be detected throughout the oocyte cytoplasm. In the overlay of the two signals (E), the ratio of green to red is substantially greater in the lateral and anterior sides of the nucleus than elsewhere in the cytoplasm, indicating that Imp is specifically concentrated at these regions of the oocyte.

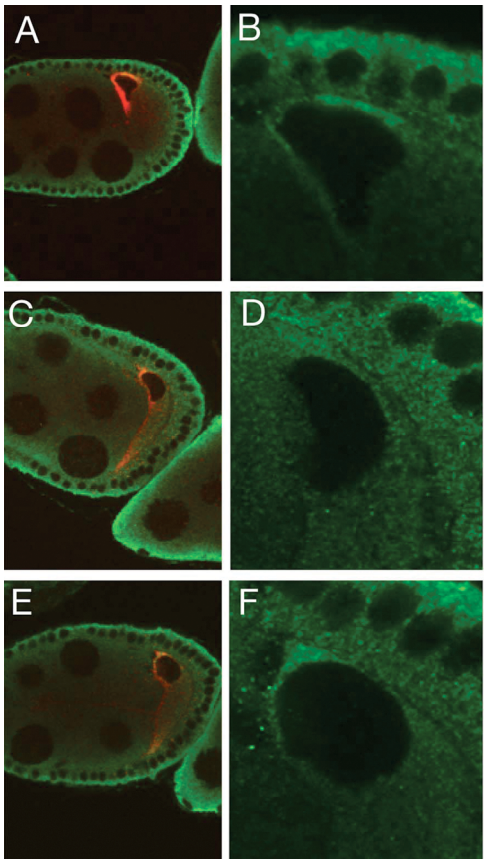
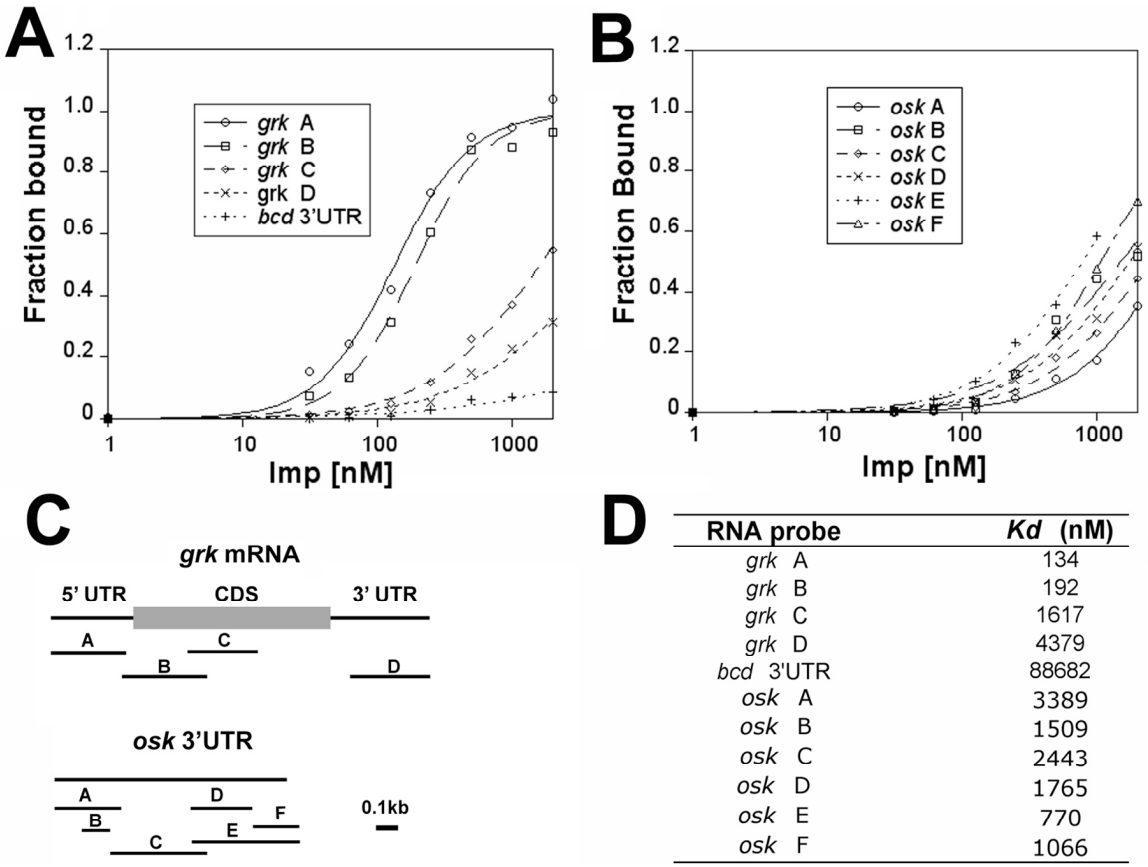


Figure 2.3 Anterodorsal localization of Imp in the oocyte is dependent on Sqd.

Panels A, C, E are stained for both Grk protein (red) and Imp protein (green). Panels B, D, F are magnifications, with only the Imp signal shown. In a wild type oocyte (A), Grk is highly concentrated adjacent to the nucleus, and Imp concentrated in a similar pattern. In *sqd* mutant oocytes (C, E) Grk protein remains at the anterior of the oocyte, but now at both dorsal and ventral positions in the optical sections. The Imp localization is greatly reduced at the dorsal surface, primarily along the lateral cortex. The residual concentration of Imp between the oocyte nucleus and the nurse cells parallels the distribution of *grk* mRNA.



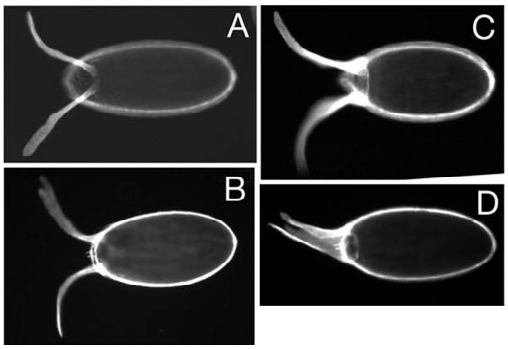
RNA probe	*Kd* (nM)
grk A	134
grk B	192
grk C	1617
grk D	4379
bcd 3'UTR	88682
osk A	3389
osk B	1509
osk C	2443
osk D	1765
osk E	770
osk F	1066

Figure 2.4 Imp binds with high affinity to *grk* mRNA.

A and B. Filter binding assays of Imp binding to portions of the *grk* mRNA (A) and *osk* mRNA 3' UTR (B). The binding assays were performed with various concentrations of Imp to allow calculation of dissociation constants.

C. Diagram of the *grk* mRNA and *osk* mRNA 3' UTR, indicating the regions used as probes.

D. Summary of dissociation constants obtained from the binding assays.



E

maternal genotype	wild type (%)	dorsalization: weak (%)	moderate (%)	strong (%)	(n)
w^{1118}	100	0	0	0	(106)
Imp^{G0072}	100	0	0	0	(74)
fs(1)K10	0	0	6	94	(51)
fs(1)K10 Imp^{G0072}	1	11	61	26	(70)

Figure 2.5 Suppression of the *fs(1)K10* phenotype by reduction of *Imp* activity.

Panels A-D are eggshells from wild type (A), or from *fs(1)K10 Imp*^{G0072} (B-D) mothers showing weak (B), moderate (C) or strong dorsalization (D). The dorsal appendages are well separated in wild type (A), and fused ventrally in the strongly dorsalized eggshells (D). Quantitation of the phenotypes is provided in E.

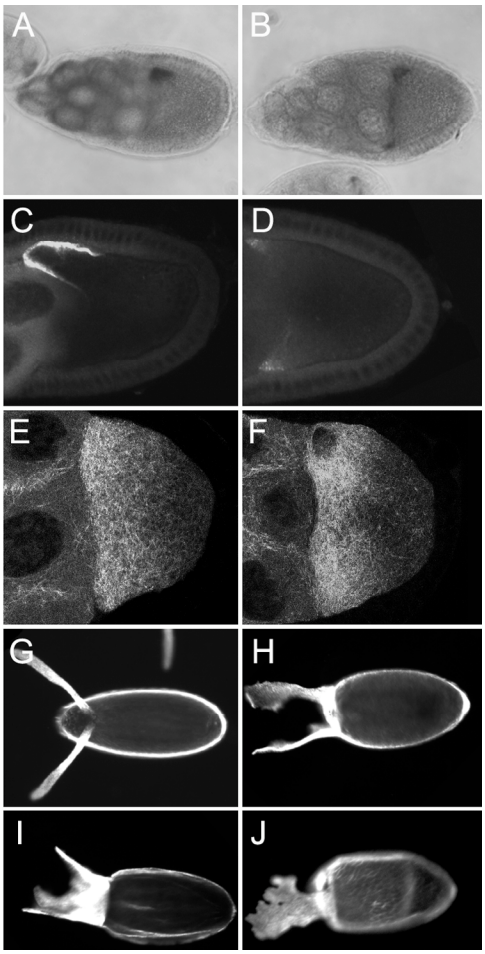


Figure 2.6 Imp overexpression alters dorsoventral patterning and regulation of *grk* mRNA.

Both *grk* mRNA (A and B) and protein (C and D) are misexpressed in Imp overexpression egg chambers. Panels A and C are wild type, while B and D express one copy of *P[UAS-Imp]* under control of the *matα4-GAL4VP16* driver.
Panels E and F show microtubule organization in live wild type (E) and Imp overexpression (F) oocytes, as detected by TauGFP. Both show a gradient of microtubule density, highest at the anterior. There are no substantial differences between the mutant and wild type.
Panels G-J show eggshells (anterior to the left) of wild type or Imp overexpression oocytes (one copy of *P[UAS-Imp]* with the *matα4-GAL4VP16* driver). All eggshells of wild type oocytes are normal (G; n=105). Imp overexpression produces eggshells (n=255) of which 17% are wild type, 20% are moderately dorsalized (H) and 58% are strongly dorsalized (I). The remaining 4% have fused but slightly expanded dorsal appendages (J).

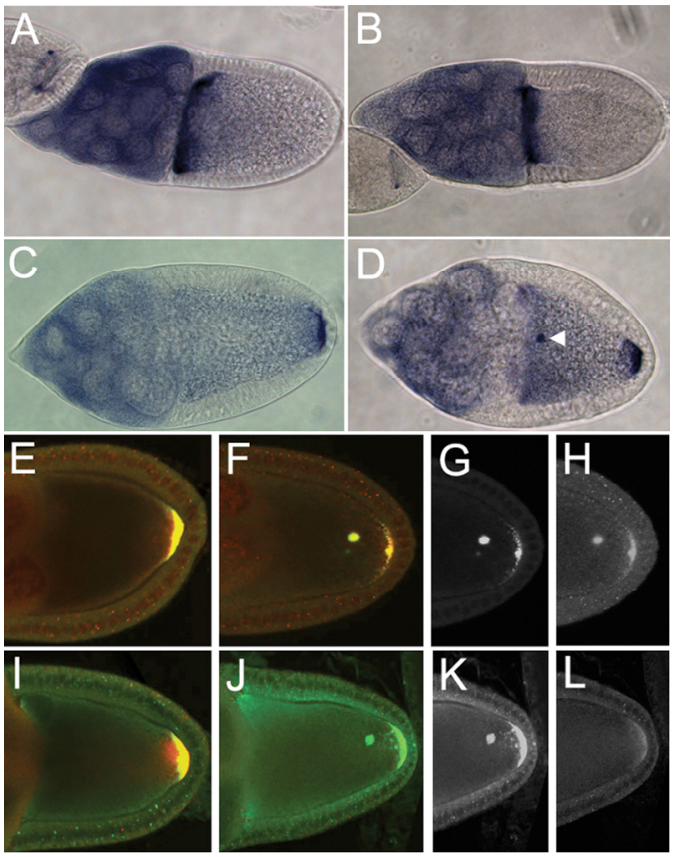


Figure 2.7 Imp overexpression disrupts polarization of the oocyte along the anteroposterior axis.

The distribution of *bcd* mRNA in wild type (A) and Imp overexpression (B) egg chambers is indistinguishable. In contrast, the posterior localization of *osk* mRNA in wild type (C) is disrupted by overexpression of Imp, with the mRNA often present in a discrete body (or rarely, bodies) in the ooplasm (arrowhead) as well as at the posterior pole (D). A similar effect is observed for Stau protein (green signal in E and F, wt and Imp overexpression, respectively), which also serves to mark the distribution of *osk* mRNA. Notably, Osk protein (red signal in E and F) also appears in the ooplasmic bodies containing *osk* mRNA and Stau protein. The Stau and Osk signals are also shown separately in panels G (Stau) and H (Osk) for clarity. The Kinesin-β-gal marker (red signal in I and J), which is heavily concentrated at the posterior pole in wild type and colocalized with Stau (green signal), is dispersed in Imp overexpression oocytes (J), with no evidence of concentration in a central zone. The Stau and Kinesin-β-gal signals are also shown separately in panels K (Stau) and L (Kinesin-β-gal) for clarity.

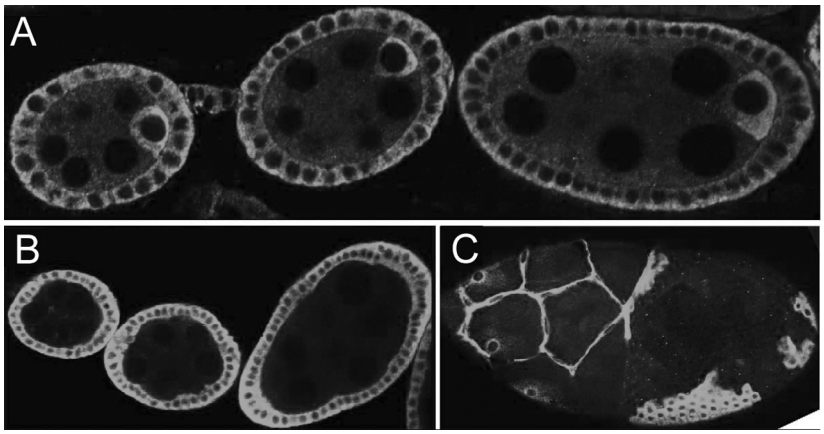


Figure 2.8 supplemental figure.

A. Early stages of oogenesis (2-6) when Imp is concentrated in the oocyte. The protein is predominantly or exclusively cytoplasmic, as it is in all cells examined. In the follicle cells Imp is initially uniform in the cytoplasm (left egg chamber), but is progressively polarized to the apical region (right egg chamber).

B-C. The Imp antibodies are specific. Using the Imp^{G0072} allele (below) egg chambers largely lacking Imp in the germline (B) or in a subset of the follicle cells (C) were produced and stained with the Imp antibody. The dramatic reduction in signal levels demonstrates that the antibody specifically detects Imp in both germline and follicle cells.

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Chapter Three: A deficiency screen to identify novel regulators of *grk* expression in a sensitized Imp overexpression background

ABSTRACT

The establishment of dorsoventral polarity in *Drosophila* eggs and future embryos relies on the precise control of *grk* mRNA/protein distribution within oocyte, in which Imp, a *Drosophila* mRNA binding protein, plays a redundant role. In order to identify other potential regulators of *grk* expression, some of which may act redundantly with *Imp*, we screened a collection of deficiencies for dominant modifiers of the dorsalized eggshell phenotype caused by Imp overexpression in germ line cells. Deletion of 12 genomic regions display dominant modification of the Imp overexpression phenotype. Further characterization of mutants of genes within these genomic regions led to identification of five modifiers, including *cyclin E (cyc E)*, *E2f transcriptional factor 1 (E2f1), lingerer (lig), snail (sna) and mushroom body expressed (mub)*. *E2f1* encodes a transcriptional factor that is involved in regulating the G1 to S phase transition during mitosis. Mutation in *E2f1* results in altered *grk* mRNA and protein distribution within oocyte, thus suggesting a role in regulation of *grk* expression.

INTRODUCTION

The establishment of dorsoventral (DV) polarity in the *Drosophila* embryo relies on the asymmetric distribution of Grk, a transforming growth factor-α-like protein, within oocyte. The restricted expression of Grk is achieved by mRNA localization and translational control (reviewed by Johnstone and Lasko, 2001; Nilson and Schupbach, 1999). During midoogenesis, *grk* mRNA is localized to the anterodorsal corner of the oocyte, where it is actively translated (Neuman-Silberberg and Schupbach, 1993, 1996). The resulting Grk protein activates *Egfr* signaling pathway in the adjacent follicle cells to establish dorsal follicle cell fates (Neuman-Silberberg and Schupbach, 1993,1996; Schupbach, 1987).

The precise spatial control of *grk* mRNA and protein expression within the oocyte is essential for DV pattern formation. A *Drosophila* RNA binding protein, *Imp*, has been implicated in this process (See Chapter two). Imp binds to *grk* mRNA specifically and co-localizes with *grk* transcripts at the anterodorsal corner of the oocyte. Mutations in *Imp* do not cause any substantial DV patterning defects, but partially suppress the dorsalization phenotype of *fs(1)k10* mutants resulting from mislocalization of Grk. Consistently, females with Imp overexpression lay dorsalized eggs, a result of mislocalized *grk* mRNA and protein. The opposing affects of overexpression and loss-of-function of *Imp* support a redundant role of *Imp* in regulation of *grk* expression.

The dominant Imp overexpression phenotype offers an opportunity to identify other potential regulators of *grk* expression, some of which may act redundantly with Imp. Here we screened a collection of deficiencies that covers about 56% of the

Drosophila genome to identify potential loci which dominantly modify the dorsalized eggshell phenotype resulting from overexpression of Imp. These deficiencies identify twelve regions that contain dominant modifiers of the Imp overexpression phenotype. Characterization of mutants of genes within the deficiencies led to identification of five modifiers, including *cyclin E (cyc E)*, *E2f transcriptional factor 1(E2f1), lingerer (lig), snail (sna),* and *mushroom body expressed (mub)*. Further analysis of these genes will be of great help in revealing their possible roles in regulation of *grk* expression.

RESULTS

Screening for deficiencies that modify the overexpression phenotype of Imp

Overexpression of Imp in the germ line cells of the ovary with the GAL4/UAS expression system (Rorth et al., 1998) results in a variety of eggshell phenotypes ranging from normal to strongly dorsalized. To obtain a sensitized background for a modifier screen, a low level of Imp was overexpressed using a maternal-α-GAL4 driver on chromosome II. Under these conditions, 78% of the resulting eggshells are normal, and the rest are either moderately (18%) or strongly dorsalized (4%, n=312, see Fig 3.2). This weak phenotype could be useful for finding modifiers, especially enhancers.

To ask whether this genetic background is appropriate for identifying interacting genes of *Imp*, we introduced strong alleles of *sqd* (Sqd has been shown to interact with Imp in a RNA dependent manner, see chapter two). The combination of Imp overexpression with sqd^{ix50}/+ results in eggs with obviously enhanced dorsalization phenotypes (15% normal, 27% weakly dorsalized and 58% strongly dorsalized, n=362, see Fig 3.2). This result validates the screen strategy.

For the screen, we used a collection of Exelixis deficiencies which cover about 56% of the *Drosophila* genome (Parks et al., 2004). The Exelixis deficiencies were generated by FRT-based deletion strategy and have several advantages over the collection of traditional deficiency mutants produced by irradiation or chemical

mutagenesis (Parks et al., 2004). First, each Exelixis deficiency deletes a smaller genomic region (140kb on average). Second, they have molecularly defined endpoints, allowing unambiguous definition of which genes are affected. Third, all the Exelixis deficiencies come from a relatively isogenic genetic background, thus reducing the chance that they harbor any pre-existing mutations, which might complicate sensitized screens. Therefore, the Exelixis deficiency collection is a better choice for a deficiency screen.

Screen results

We screened 421 deficiencies (200 on chromosome II, 221 on chromosome III) for the ability to modify the dorsalized phenotype derived from Imp overexpresion (for screen scheme, see Fig 3.1). Of all the deficiencies screened, 11 showed consistent enhancement of the Imp overexpression phenotype (4 on chromosome II, 7 on chromosome III, see Table 1), while one genomic region displayed "suppression" of the dorsalized phenotype (the implications of suppression are considered below). Based on the degree of enhancement, the 11 enhancers were grouped into three categories: strong enhancers (5), moderate enhancers (4) and weak enhancers (2). All these modifiers were examined in detail and will be discussed individually in the following part of the chapter.

***Df(2L)Exel 7063* (strong enhancer)**

Df(2L)Exel 7063, which removes the interval between 35D2 and 35D4, strongly enhances the dorsalized phenotype resulting from Imp overexpression. Among the

twenty-two genes in this region, four of them have known characterized mutations, while three are associated with P element insertions with no known effect on gene expression. Available mutations or associated P element insertions of those genes were tested for modification of the Imp overexpression phenotype (see Table 2). Mutations in *snail (sna)* and *cyclin E (cycE),* both contribute to the modification of the Imp overexpression phenotype but to different extents. *sna* encodes a zinc finger transcriptional repressor that is required in mesoderm formation during embryogenesis (Hemavathy et al., 1997; Kosman et al., 1991; Ip et al., 1992; Alberga et al., 1991; Leptin, 1991). The loss-of-function sna^{18} allele (Ray et al., 1991) moderately enhances the Imp over-expression phenotype, which suggests that *sna* might have a role in regulating *grk* expression during oogenesis. As the sna^{18} allele is embryonic lethal, mutant clonal analysis of this allele in germ line cells will be required to examine its potential ovarian phenotype.

CycE is a G1 cyclin that promotes the G1 to S transition during mitosis (Richardson et al., 1995; Knoblich et al., 1994). Two alleles of *cycE*, $cycE^{05206}$ (hypomorph) and $cycE^{AR95}$ (null), display strong enhancement of the Imp overexpression phenotype. Efforts to reveal the role of *cycE* in DV polarity formation will be discussed later in this chapter.

***Df(3R)Exel 6186* (strong enhancer)**

Df(3R)Exel 6186, which deletes the genomic region between 93E6 and 93F1, is a strong enhancer. Among the five genes residing in this region, only *E2f1* has existing mutations whose phenotypes are already characterized. Of the two available *E2f1* alleles

tested, *E2f1*^{07172}, is a loss-of-function allele and displays the same enhancement of the Imp overexpression phenotype as *Df(3R)Exel 6186* (See Table 3). Thus *E2f1* might interact with *Imp* to regulate the localized expression of *grk* during oogenesis. *E2f1* encodes a transcriptional factor that is required for G1/S transition during mitosis (Duronio et al., 1995; Royzman et al., 1997). Additional studies of this gene will be discussed later in this chapter.

***Df(2L)Exel 8038* (strong enhancer)**

Df(2L)Exel 8038, which removes the genomic region between 36D3 and 36E3, is a strong modifier. Another deficiency, *Df(2L)Exel 7070*, with breakpoints between 36D2 and 36E1, does not affect the Imp overexpression phenotype, which limits the position of the modifier to 36E1 to 36E3 (see Table 5). There are fifteen genes in this genomic region: five have existing mutations whose phenotypes are already known and three are associated with P element insertions with no known effect on gene expression. Of the five genes with characterized mutations, four were tested for modification of the Imp overexpression phenotype; of the three genes that are associated with P element insertions, two were tested. However, none of the tested individual mutants or P element insertions from this region display enhancement of the Imp overexpression phenotype (see Table 4).

***Df(3R)Exel6274* (strong enhancer)**

Df(3R)Exel 6274, which removes the genomic region between 94E4 and 94E11, is a strong enhancer. Another overlapping deficiency, *Df(3R)Exel 9012,* with breakpoints between 94E9 and 94E13, is a moderate enhancer. However, a third overlapping deficiency, *Df(3R)Exel 6280,* the deletion region (94E5-94E11) of which covers the shared region of the above two deficiencies, fails to modify the Imp overexpression phenotype (see Table 7). There are two possible explanations for these results. First, *Df(3R)Exel 6280* may not delete the region as originally described. As 8% of the Exelixis deficiency collection do not actually contain the deficiency assigned to them (http://flystocks.bio.indiana.edu/Browse/df-dp/exel-dfs.htm), it would not be surprising if *Df(3R)Exel 6280* falls into that category. Second, there might exist two modifiers: one resides in the 94E4-94E5 region and accounts for the strong enhancement by *Df(3R)Exel 6274*, while another one falls in the region between 94E11 and 94E13 and is responsible for the moderate enhancement by *Df(3R)Exel 9012*. However, there is only one gene, *pointed (pnt),* in the 94E11-94E13 region. Four characterized mutations of *pnt*, including two amorphs, do not modify the Imp overexpression phenotype, which excludes the second possibility. Therefore *Df(3R)Exel 6280* may not remove the described region.

There are seventeen genes within the genomic region 94E4-94E11; four have characterized mutations; seven are associated with P element insertions with unknown effect on gene expression. Of the four genes with existing mutations, three were tested for modification of the Imp overexpression phenotype; of the seven genes with P element

insertions, two were tested. However, none of the tested mutations or P element insertions alters the Imp overexpression phenotype (see Table 6).

Df(3L)Exel 6137 **(moderate enhancer)**

Df(3L) Exel6137, which deletes the genomic region between 78F4 and 79A4, is an moderate enhancer. An overlapping deficiency, *Df(3L)ED4968*, which removes the genomic region between 78D5 to 79A2, displays a similar degree of enhancement (see table 9). Therefore, these two deficiencies limit the region responsible to 78F4–79A2. There are two genes in this region, CR32449 and *mushroom body expressed* (*mub*). CR32449 does not have any existing mutations or associated P elements. A lethal P element insertion in *mub, mub*^{04093}, is an enhancer of the dorsalized phenotype caused by Imp overexpression (see Table 8), which indicates that *mub* genetically interacts with *Imp*. More research on the *mub* gene will be discussed later in this chapter.

Df(2R)Exel 6056 **and** ***Df(2R)Exel 7094*** **(moderate enhancers)**

Two overlapping deficiencies, *Df(2R)Exel 6056* and *Df(2R)Exel 7094*, which delete genomic regions 44A4-44C2 and 44A4-44B4, respectively, are moderate enhancers. Two additional deficiencies, *Df(2R)Exel 6057* and *Df(2R)Exel 7095*, remove the regions 44B9-44C4 and 44B3-44C2 respectively, and are not modifiers. Thus the genomic region required for modification of the Imp overexpression phenotype is confined to 44A4-44B3 (see Table 11), assuming that all deficiencies contain the assigned deletions. There are fifteen genes in this genomic region: four have known

characterized mutations, ten are associated with P element insertions with no indication that they disrupt gene function. Of the four genes with existing mutations, three were tested for modification of the dorsalization phenotype arising from Imp overexpression; of the ten genes that are associated with P element insertions, seven were tested.

One mutant from this genomic region, *l(2)SH1919*^{SH1919}, a lethal P element insertion in the *lingerer (lig)* gene, shows a similar enhancement of the Imp overexpression phenotype (see Table 10). *lig* encodes a cytoplasmic protein expressed in the central nervous system (CNS), imaginal discs and gonads (Kuniyoshi et al., 2002). Lig protein belongs to a family of proteins that have five conserved domains with no previously known function. *lig* mutant flies are defective in initiation and termination of copulation, but with no obvious abnormalities in their genitalia (Kuniyoshi et al., 2002). Therefore, *lig* might act in the nervous system to control mating behavior during courtship.

The enhancement of the Imp overexpression phenotype by the *lig* mutation suggests that *lig* might function in the ovary to regulate *grk* expression. It will be interesting to know whether *lig* mutants alone display any ovarian phenotype. However, the available *lig* allele, *l(2)SH1919*^{SH1919}, is lethal when homozygous. Clonal analysis of this allele in germ line cells of the ovary will be necessary to ask if *lig* acts in dorsoventral patterning.

***Df(2R)Exel 7096* (moderate enhancer)**

Df(2R)Exel 7096, which removes the interval between 44C6 and 44D3, is a moderate enhancer. Another deficiency overlapping this region, *Df(2R)Exel6058,* with

breakpoints between 44C4 and 44D1, is not a modifier, which confines the genomic region responsible for modification of the Imp overexpression phenotype to 44D1-44D3 (see Table 12), assuming that the deficiency contains the reported deletion. There are fifteen genes in this genomic region, none of which have known characterized mutations. Five of these genes are associated with P element insertions with no known effect on gene expression. However, no P element insertions within this region have been tested for the ability to modify the Imp overexpression phenotype.

***Df(3R)Exel 7313* (moderate enhancer)**

Df(3R)Exel 7313, with breakpoints between 87A9 and 87B3, is a moderate enhancer. Unexpectedly, another deficiency, *Df(3R)Exel6162*, which removes the genomic region between 87A1 and 87B5, including the region covered by *Df(3R)Exel 7313*, has no effect on Imp overexpression background. In addition, a third overlapping deficiency, *Df(3R)Exel 7314*, which removes the region between 87B3 and 87B8, also fails to modify the Imp overexpression phenotype (see Table 13).

One explanation for these conflicting results is that some of these deficiencies may not remove the described region. To exclude this possibility, further analysis such as complementation tests or southern blot will help to confirm the breakpoints of these questionable deficiencies. However, if they do delete the regions as described, the region could include an enhancer and a repressor, which together would not appear to act as modifiers. For this case, generating smaller deficiencies flanking these two regions will

facilitate the refinement of the genomic regions required for modification of the Imp overexpression phenotype.

Df(3R)Exel 6191 **(weak enhancer)**

Df(3R)Exel 6191, with breakpoints between 94A9 and 94B2, is a weak enhancer. There are thirty-seven genes within this genomic region. No characterized mutations or P element insertions in this region have been examined in the Imp overexpression background yet.

Df(3R)Exel6203 **(weak enhancer)**

Df(3R)Exel6203, which deletes the genomic region between 96E2 and 96E6, is a weak enhancer. There are eight genes within this region, including *musashi (msi),* CG4582, CG12250, CG4673, CG5079, CG5071, CG4685 and CG17383. *msi* encodes an RNA binding protein involved in translational repression of Tramtrack69 (TTK69), a zinc-finger transcriptional repressor (Nakamura et al., 1994; Okabe et al., 2001). The null allele of *msi, msi*^{1}, is a weak enhancer. Another *msi* allele, *msi*^{2}, also enhances the Imp overexpression phenotype, but to a lesser extent than *msi*^{1}. *msi*^{1} /*msi*^{2} flies are fertile and have no overt ovarian phenotype (see Table 14).

For the other seven genes in this region, none have characterized mutations, but two of them are associated with P element insertions with unknown effect on gene expression. We tested one viable P element insertion in CG17383, *CG17383*^{BG00794}, in Imp overexpression background. It shows weaker enhancement of the Imp

overexpression phenotype than *msi*[1]. Since it remains unknown whether *CG17383*^{BG00794} affects expression of CG17383, it is likely that both *msi* and CG17383 contribute to the modification of the Imp overexpression phenotype, or the effect of *CG17383*^{BG00794} in Imp overexpression background is due to some unidentified background mutation in the *CG17383*^{BG00794} chromosome. Generating strong mutations of CG17383 and other genes in this region, as well as cleaning up the *CG17383*^{BG00794} chromosome might facilitate the identification of candidate gene(s) responsible for modification of the Imp overexpression phenotype.

***Df(3R)Exel 6164 (Df(3R)Exel 6165) and Df(3R)Exel 7316* ("suppressors")**

Unlike other modifiers of the Imp overexpression phenotype, *Df(3R)Exel 6164* and *Df(3R)Exel 6165*, which both delete the region between 87B5 and 87B10, dominantly modify the Imp overexpression phenotype in an unusual way. Imp overexpression females lay eggs of which 81% have wild type eggshells (n=190), 18% are either moderately or strongly dorsalized, and less than 1% have fused dorsal appendages, a typical weakly ventralized phenotype. However, in the presence of *Df(3R)Exel 6164*, only 36% of the eggs laid by Imp overexpression females are wild type (n=208), 5% have either moderately or strongly dorsalized eggshells, whereas 33% have dorsal appendages that are fused or very close at the base, and 26% have fused but slightly expanded dorsal appendages (a combination of dorsalized and ventralized phenotypes, which might result from the reduced but mislocalized Grk protein within the oocyte, see Fig 3.3 and table 17). Therefore, the dorsalization phenotype resulting from

Imp over-expression does not appear to be significantly enhanced or suppressed by the presence of *Df(3R)Exel 6164*. Instead, it is the rarely observed ventralization phenotype that is substantially enhanced, which implicates a reduction of Grk protein level in the corresponding oocytes. It remains to be investigated whether the level and distribution pattern of *grk* mRNA/protein are altered in these oocytes.

Another overlapping deficiency, *Df(3R)Exel 7316*, with breakpoints between 87B9 and 87B11, also displays similar modification of the Imp overexpression background. Therefore, these two deficiencies refined the genomic region responsible for modification of the Imp overexpression phenotype to region 87B9-87B10 (see Table 16). There are sixteen genes within this region: one (Ppl-87B) has known characterized mutations; nine are associated with P element insertions with no known effect on expression level. We tested modification of the Imp overexpression phenotype with characterized mutations from Ppl-87B, as well as P element insertions from four other genes within that genomic region. However, none of them displays modification of the Imp overexpression phenotype (see Table 15). Further experiments such as generating smaller deficiencies spanning this region and identification of strong alleles of candidate genes within this region, will facilitate the identification of gene(s) responsible for the modification of the Imp overexpression phenotype.

Initial functional analysis of *Drosophila mushroom-body expressed (mub)*

mushroom-body expressed (*mub*) encodes an RNA binding protein with three evolutionary conserved KH (hnRNP K homology) domains, an RNA binding motif found

in many RNA binding proteins (Grams and Korge, 1998). Mub is very similar to a group of human poly (rC)-binding proteins, PCBP1-4, especially in the KH domains. Taking PCBP-2 for example, the identity of individual KH domain between PCBP-2 and Mub ranges from 60.7% to 69.8% (Similarity ranges from 79.7% to 87.9%)(Grams and Korge, 1998; Makeyev and Liebhaber, 2002). PCBP proteins are predominantly cytoplasmic and function in mRNA stability, translational control and apoptosis induction (reviewed by Makeyev and Liebhaber, 2002). As the *Drosophila* ortholog of PCBPs, *mub* might also function in these cellular processes.

mub expression pattern during oogenesis and embryogenesis

Similar to *Imp, mub* displays a biphasic expression pattern during embryogenesis (Tomancak et al., 2002; Grams and Korge, 1998). In early stages of development, *mub* mRNA is distributed evenly in embryos, suggesting that the *mub* transcripts are synthesized during oogenesis and deposited into eggs (Tomancak et al., 2002). After stage 3, the maternally derived *mub* mRNA disappears (Tomancak et al., 2002). At later stage of embryogenesis, strong zygotic transcription of *mub* is detected in mushroom body, a neural structure essential for learning and memory, where *mub* transcripts are actively translated into protein (Tomancak et al., 2002; Makeyev and Liebhaber, 2002).

Modification of the Imp overexpression phenotype by the *mub* mutation strongly indicates a role of Mub during oogenesis. To explore it further, we examined the subcellular distribution of Mub in ovaries by immunostaining. Similar to its human orthologs, Mub is predominantly cytoplasmic. However, no obvious subcellular

enrichment is observed in the ovary, which could result from poor immunoreactivity of the antibody or staining conditions. Therefore, an alternative approach was employed, in which a MubGFP fusion protein was expressed in germ line cells with the GAL4/UAS expression system. When expressed with a maternal-α-GAL4 driver (on Chromosome II), MubGFP is detected in particles dispersed in the cytoplasm of the nurse cells and oocyte (Fig 3.5), in a pattern similar to sponge body components (Wilsch-Brauninger et al., 1997). The presence of MubGFP in sponge bodies is further confirmed by colocalization with the sponge body component Bru (unpublished data, M.Snee). Beyond its enrichment in sponge bodies, MubGFP is concentrated at the anterior margin of the oocyte during stage 8-9A. At stage 9B-10, MubGFP is localized at the posterior pole of the oocyte, where *osk* mRNA is normally localized, which suggests that Mub might play a role in regulation of *osk* expression (Fig 3.5).

Phenotypic analysis of *mub* loss-of-function mutant

The dominant modification of the Imp overexpression phenotype by *mub* mutations strongly suggests that *mub* might interact with *Imp* to regulate localized *grk* expression. It will be interesting to know whether *mub* mutations alone will result in any defects in dorsoventral patterning.

mub^{04093}, a P element insertion near the first exon of *mub* (Fig 3.4), is recessive lethal. However, some mub^{04093} hemizygous ($mub^{04093}/Df(3L)Exel\ 6137$) flies do occasionally eclose, which suggests that the recessive lethal phenotype of mub^{04093} might result from another mutation on the mub^{04093} chromosome. These hemizygous flies

display defective locomotive behavior (they are flightless and prone to getting stuck in the growth medium) and usually die within 3 days. The few eggs laid by female flies are phenotypically normal. In *mub*^{04093}*/Df(3L)Exel 6137* flies, the Mub protein level is greatly reduced in comparison with wild type flies, as revealed by western blot analysis (data not shown). Therefore, the defective locomotive behavior of *mub*^{04093}*/ Df(3L)Exel 6137* flies might be attributed to the reduction of Mub protein levels in these flies.

Another deficiency, *Df(3L)ED 4968*, with breakpoints between 78F4 and 79A2, deletes the first exon and part of the first intron of *mub*, but leaves the entire coding region intact (Fig 3.4). *mub*^{04093}*/Df(3L)ED4798* flies lay eggs with no overt phenotype. These flies are healthier than *mub*^{04093}*/ Df(3L)Exel 6137* flies, as they display less severe locomotive behavior and have a longer life span (2-7 days), which suggests that *mub* might still be transcribed from a hidden transcription start point following the deleted region in *Df(3L)ED4798*, but with much lower efficiency. Therefore, it will be interesting to know whether the Mub protein levels are higher in *mub*^{04093}*/Df(3L)ED4798* flies than *mub*^{04093}*/Df(3L)Exel 6137* flies.

The phenotypic analysis of *mub*^{04093} fails to reveal any overt ovarian phenotype of *mub*, which could result from any of the following reasons. First, this *mub* allele may not be strong enough to cause any defects in oogenesis. Second, *mub* might play a redundant role in regulating *grk* expression. Third, *mub* might not act in *grk* regulation. Therefore, generating null alleles of *mub* will be of great help to differentiate these possibilities. There are several transposons containing FRT sites scattered throughout the *mub* gene region, which can be employed to generate *mub* deletions with the FRT-based deletion

strategy (Thibault et al., 2004; Parks et al., 2004). The basis for this strategy is stated as follows. In the presence of FLP recombinase, efficient trans-recombination occurs between two FRT elements on homologous chromosomes, which will result in a deletion that removes the genomic region between these two FRT sites. Unlike deletions made by traditional methods, deletions generated by this FRT-based deletion strategy have molecularly defined endpoints, which will greatly facilitate mutation mapping. In some cases the deletions generated by this strategy can be easily spotted by loss of the *w+* markers associated with the transposons, if the transposons are oriented in the genome such that the w+ markers are positioned between the FRTs and thus deleted in the course recombination (Thibault et al., 2004; Parks et al., 2004).

Two pairs of FRT-containing transposons have been chosen to generate *mub* null alleles separately. The first pair is mub^{d01601} and mub^{e01336}, both of which flank the *mub* gene. A deletion generated by this pair will remove the entire *mub* gene (Fig 3.4). As *mub* spans a 47 kb genomic region, it is possible that some unidentified genes might exist in this large region. To minimize the chance of removing unnecessary sequences, we choose another pair of transposons, mub^{e01971} and mub^{d09454}, which reside in intron 1 and intron 7, respectively. A deletion produced by this pair will only remove a 12kb genomic region, including the first 217 amino acids of Mub protein (the full length protein is 386 aa), thus leaving the most of the *mub* genomic region intact. A comparison of the phenotypes of these two *mub* nulls will provide a clue as to whether there are other essential genes residing in this 47 kb genomic region.

The abundance of FRT-containing transposon insertions in the *mub* gene has facilitated the deletion analysis of the conserved domains of Mub protein. A pair of transposons, mub^{d03166} and mub^{e01336}, were chosen to make small deletion which will result in a truncated Mub protein with the first two KH domains left. By comparing the phenotype of this deletion and *mub* null alleles, we will know whether the third KH domain is indispensable for Mub function.

The deletions generated by the above three transposon pairs can be identified by the loss of a *w+* marker (Parks et al., 2004). So far, we have obtained 4-7 individual deletion lines for each transposon pair based on the eye color. In general, four out of five w- individual progeny should remove the desired genomic region, as reported by Parks et al (Parks et al., 2004). Thus, we should have at least two or three authentic deletion lines for each transposon pair. PCR or Southern blot analysis will be required to confirm that the expected deletions are present,

Phenotypic analysis of *mub* gain-of-function mutant

As a complementary approach to explore *mub's* role during oogenesis, MubGFP (or Mub) was overexpressed in germ line cells with the GAL4/UAS system. Most eggs laid by MubGFP (or Mub) overexpression females have open-ended chorions ("cup-shaped"), as the anterior end of the eggshell fails to close (Fig 3.6). This phenotype is also observed in *cup, BicC, quit, chalice* and *kelch* mutants and results from a failure of centripetal follicle cell migration in these mutants egg chambers (Schupbach and Wieschaus, 1991). In the cup-shaped eggs, the two dorsal appendages are missing, or

form a blob-like structure located at a lower position of the eggshells, or form a single but slightly wide dorsal appendage, or are fused but widely expanded (Fig 3.6). The dorsal appendage defects indicate that axial patterning, both anteroposterior and dorsoventral, is defective. As Grk is essential for proper specification of both axes during oogenesis, it will be interesting to know whether the level and distribution pattern of *grk* mRNA/protein are disrupted in MubGFP (or Mub) overexpression oocytes.

cyclin E (cycE)

Drosophila cycE encodes a mitotic cyclin that forms a complex with the cyclin-dependent kinase Cdk2 to control the transition from G1(G) phase to S phase during mitosis and endocycle, a simplified version of mitosis in which cell replicates its DNA without division (Vidwans and Su, 2001; Dulic et al., 1992; Koff et al., 1992; Knoblich et al., 1994; Richardson et al., 1995). Strong *cycE* mutations block entry into S phase (Knoblich et al., 1994), while ectopic expression of *cycE* induces cells to enter S phase prematurely (Knoblich et al., 1994; Richardson et al., 1995).

In addition to its role in cell cycle control, *cycE* is also involved in cell fate determination. In the central nervous system, *cycE* is both necessary and sufficient to specify the thoracic identity of NB6-4t (t, thoracic) neuroblast (Berger et al., 2005). This role does not require components of the cell cycle machinery. In addition, in eggchambers from females that are homozygous for a hypomorphic *cycE* mutation, $cycE^{01672}$, more than one germ cell differentiates into oocytes, which indicates that *cycE* is involved in oocyte determination (Lilly and Spradling, 1996).

As described earlier, two *cycE* alleles, $cycE^{05206}$ and $cycE^{AR95}$, strongly enhance the dorsalized phenotype caused by Imp overexpression. In Imp overexpression ovaries that are heterozygous for either of these two *cycE* alleles, nurse cells are present in appropriate numbers and nuclear morphology (as revealed by DAPI staining) is normal for nurse cells and oocytes, suggesting that cell proliferation is normal. In contrast, Grk mislocalization to the anterior region is enchanced in these oocytes, to a degree higher than in Imp overexpression oocytes. Therefore, *cycE* might have a role in regulation of localized *grk* expression. It is uncertain if the role of cycE in modification of Imp overexpression phenotype is related to its role in cell cycle control.

To further explore the potential roles of *cycE* in regulation of *grk* expression, phenotypic analysis were performed with the available *cycE* alleles, including $cycE^{AR95}$, $cycE^{KG07848}$, $cycE^{KG00239}$, $cycE^{05206}$ and $cycE^{k05007}$. Except for $cycE^{KG07848}$, the *cycE* alleles are homozygous lethal. In addition, flies that are transheterozygous for any two of the four alleles are not viable (Table 18). $cycE^{KG07848}$ is a semilethal P element insertion near the first exon of the *cycE* gene. Flies homozygous or hemizygous for $cycE^{KG07848}$ lay eggs which have very thin and fragile chorions (Fig 3.7), suggesting defects in chorion gene amplification during follicle cell endoreplication, in which *cycE* plays an essential role (Calvi et al., 1998). The dorsal appendages of these eggs are also very thin and fragile, which makes it hard to detect any weak dorsoventral polarity defects. Mutant analysis of these *cycE* alleles in germline clones will shed light on the potential roles of *cycE* in dorsoventral polarity determination. As *cycE* is required for mitosis and the endocycle in germline cells, strong *cycE* mutations will arrest oogenesis at an early stange. To solve

this problem, ovoD and histone GFP can be used as markers to generate mosaic eggchambers, in which all or some germline cells homozygous for the above *cycE* alleles. For strong *cycE* alleles, the partial *cycE* activity in mosaic eggchambers with some of the germ line cells mutant for *cycE* alleles might allow oogenesis to proceed through late stages, which will facilitate our analysis of dorsoventral polarity defects.

Some eggs produced by females that are homozygous or hemizygous for $cycE^{KG07848}$ have extra dorsal appendage(s) at random positions on the eggshells (Fig 3.7), indicating the presence of ectopic Grk signaling in these eggchambers. As Grk is usually associated oocyte nucleus, and weak *cycE* mutation results in more than one oocyte nuclei in a single eggchamber (Lilly and Spradling, 1996), the ectopic Grk signaling might result from the existence of more than one oocyte nuclei in these mutant eggchambers. It remains to be investigated whether multiple oocyte nuclei do exist in the $cycE^{KG07848}$ mutant oocytes.

E2f1

The transition from G1 gap to S phase is a critical point for cell cycle regulation (reviewed by Pardee, 1989; Sherr, 1994). In mammals and *Drosophila*, the G1-S transition is regulated by E2F transcriptional factors. In response to cell proliferation signal, E2f activates the transcription of a wide variety of downstream target genes required for S-phase, including cell cycle regulators (e.g. Cyclin E, Cyclin A, cdks) and genes required for DNA replication (e.g. RNR, DHFR, ORC1, CDC6, MCMs, DNApol α etc) (DeGregori et al., 1997; Vigo et al., 1999). In addition, E2F can repress the

transcription of some of these target genes during G1 phase by binding to a pocket protein (a family of tumor suppressors), such as the retinoblastoma protein (RB) (Weintraub et al., 1995; Zwicker et al., 1996). The association of pocket proteins with E2F is largely regulated by G1 cyclin/cyclin dependent kinase (i.e. cyclinD/cdk4 and cyclinE/cdk2) mediated phosphorylation. E2F binds with high affinity to the hypophosphorylated form of pocket protein, but with low affinity to the phosphorylated form (Harbour et al., 1999).

E2F is a transcriptional heterodimer composed of two subunits, E2f and DP (Duronio et al., 1995; La Thangue, 1994; Nevins, 1992; Slansky and Farnham, 1996). In mammals there are six E2f family members and two DP family members (for review, see Trimarchi and Lees, 2002). In *Drosophila*, only two *E2f* genes (*E2f1 and E2f2*), one *DP* gene and one pocket protein (RBF) have been identified (Dynlacht et al., 1994; Hao et al., 1995; Ohtani and Nevins, 1994; Sawado et al., 1998), which simplifies the functional analysis of E2F transcriptional factors. Strong mutations in *E2f1* result in proliferation defects and a failure to enter into S phase, while overexpression of E2f1 in embryos and imaginal discs causes premature entry into S phase (Asano et al., 1996; Du et al., 1996; Duronio et al., 1996).

The loss of function allele of *E2f1, E2f1*^{07172}, strongly enhances the dorsalized phenotype caused by Imp overexpression. In oocytes from Imp overexpression females that are heterozygous for *E2f1*^{07172}, the occurrence of mislocalized Grk at the anterior region is more frequent than the Imp overexpression background, which suggests *E2f1* might play a role in regulation of localized *grk* expression.

To investigate the role of E2f1 in dorsoventral patterning, phenotypic analysis was performed with the $E2f1^{07172}$ and $E2f1^{i2}$ alleles. $E2f1^{07172}$ is recessive lethal. Germline clones of $E2f1^{07172}$ arrest early in oogenesis, which complicates the phenotypic analysis of this allele in later oogenesis. Another *E2f1* allele, $E2f1^{i2}$, produces a truncated E2f1 protein with the transcriptional activation domain absent (Du, 2000). $E2f1^{i2}$ homozygous flies are relatively smaller in body size and have rough eyes. The female flies of this genotype are sterile, with oogenesis arrested at an early stage. However, $E2f1^{07172}/E2f1^{i2}$ females are fertile. The eggs laid by these females display a variety of dorsoventral polarity defects (Fig 3.8I-M), including eggshells with fused dorsal appendages (ventralized, 22%), eggshells with fused but slightly expanded dorsal appendages (between dorsalized and ventralized, 39%), and eggshells with fused but widely spread dorsal appendages (strongly dorsalized, 11%, N=130). In addition, 8% of the eggs laid by $E2f1^{07172}/E2f1^{i2}$ females have greatly degenerated dorsal appendages, which obscures the dorsoventral polarity pattern of these eggshells.

Consistent with the dorsoventral defects of the eggshells, the Grk distribution pattern in oocytes from $E2f1^{07172}/E2f1^{i2}$ females is slightly altered (Fig 3.8F, H). In 66% of the stage 9 oocytes (N=38), Grk protein is reduced at the dorsal corner and slightly spreads to the ventral side, instead of being restricted to the anterodorsal corner. As oogenesis proceeds, this defect becomes more penetrant, with 72% of the stage 10 mutant oocytes (N=18) have mildly mislocalized Grk, whereas Grk localization is completely normal in wild type oocytes at both stages. In situ analysis of *grk* mRNA indicates that the altered Grk distribution pattern might stem from a mild *grk* mRNA localization

defect: in some midstage oocytes from $E2f1^{07172}/E2f1^{i2}$ females, *grk* mRNA slightly extends towards the ventral side, rather than forming a crescent adjacent to the oocyte nucleus at the anterodorsal corner. In addition to the anterodorsal localization defects, *grk* mRNA is detected at both anterior and posterior poles of the mutant oocyte at stage 7, instead of forming an anterior ring at the anterior margin as in wilytype oocytes (Fig 3.8B). One explanation for this defect is the translocation of *grk* mRNA from the posterior pole to the anterior cortex is delayed in mutant oocytes, which could result from defective MT reorganization at stage 7. It remains to be investigated whether the anteroposterior axis is also affected in *E2f1* mutants, and in which cell type *E2f1* is required for the establishment of dorsoventral polarity.

DISCUSSION

The establishment of dorsoventral polarity in *Drosophila* eggs and future embryos depends on the precise control of *grk* mRNA and protein distribution during oogenesis, in which Imp, a Drosophila mRNA binding protein plays a redundant role. Here we have conducted a sensitized deficiency screen to identify genes that are candidates to be novel regulators of *grk* expression. Deletions of 12 genomic regions display dominant modification of the dorsalized phenotype derived from Imp overexpression. Five genes within four of these regions have been verified to be responsible for the dominant modification of the Imp overexpression phenotype. Among these identified genes, *cycE* and *E2f1* mutants are strong enhancers, while *lig, sna* and *mub* mutants display moderate enhancement of the Imp overexpression phenotype.

Does *mub* have a role in regulation of *grk* or *osk* expression?

In this study, we have demonstrated that *mub* mutations moderately enhance the dorsalized phenotype caused by Imp overexpression. This effect was mediated through alteration of the distribution pattern of *grk* mRNA/protein, as more mislocalized Grk at the anterior cortex is detected in oocytes from Imp overexpression females that are heterozygous for *mub* mutation. Therefore, *mub* might function in regulation of localized *grk* expression during oogenesis.

The structure similarity between *mub* and other members of PCBP family provides some clues to its function. The PCBP proteins have been shown to be involved in mRNA stability, translational control and apoptosis induction (for review, see

Makeyev and Liebhaber, 2002). As a PCBP family member, *mub* might have a similar function. The dominant enhancement of the mild Grk mislocalization in the Imp overexpression background by *mub* mutation rules out the possibility that *mub* functions in stabilization of *grk* transcripts. If *mub* were to stabilize *grk* mRNA, then reduction of *mub* activity should cause a reduction in *grk* activity, which is not what was observed. In addition, the *mub* mutation does not induce or suppress the apoptosis of germ line cells in the Imp overexpression females. Therefore, if *mub* does have a role similar to that of PCBP proteins, it is more likely to play a role in regulation of *grk* translation. Other evidence supporting the role of *mub* in *grk* translation comes from yeast two hybrid experiments (Giot et al., 2003). Bruno, a well known translational repressor of *osk* and *grk* transcripts (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995), has been shown to interact with Mub in this assay. Thus Mub might co-operate with Bruno to regulate *osk* and *grk* translation. Since yeast two hybrid assay sometimes produce false positives, more direct assays, such as coimmunoprecipitation or GST pull downs, should be performed to confirm the physical interaction between Mub and Bruno.

When MubGFP is expressed in germ line cells, it is concentrated at the posterior pole of the oocyte during stages 9B-10, where *osk* mRNA is localized. The co-localization of MubGFP and *osk* transcripts indicates that *mub* might also have a role in regulation of *osk* expression. To investigate the potential role of Mub in *osk* expression, the following questions remains to be answered. First, does the posterior concentration of Mub require *osk* localization machinery? Second, does Mub bind to *osk* mRNA specifically? Third, is the distribution pattern of *osk* mRNA disrupted in loss-of-function

or gain-of-function mutants? It would be of no surprise if *mub* functions in regulation of both *grk* and *osk* expression, as these two transcripts share some components during localization and translation processes.

The role of *E2f1* and *cycE* in regulation of dorsoventral polarity determination

The identification of *cycE* and *E2f1* as strong dominant enhancers of the Imp overexpression phenotype is quite surprising, as both genes are well known as essential regulators of the G1 to S phase transition during mitosis or endoreplication, and no cell proliferation defects are detected in Imp overexpression eggchambers. Enhancement of the Imp overexpression phenotype by *cycE* and *E2f1* mutations could be an indirect result of cell cycle progression defects, or reflect a novel role of *cycE* or *E2f1* in regulation of *grk* expression which is independent of cell cycle control.

In *Drosophila* ovaries, each eggchamber contains 16 interconnected germ cells, which arise from a progenitor cell, the cystoblast, after four synchronous mitotic cycles with incomplete cytokinesis (Spradling, 1993). One of the 16 cysts with four ring canals becomes the oocyte and arrests in prophase I of meiosis. The other 15 cysts develop as nurse cells and enter the endoreplication cycle, a modified version of mitosis in which cell alternates between G and S phase without intervening mitotic phase. Consequently, nurse cells increase their DNA content dramatically and become polyploid. In ovaries of $E2f1^{07172}/E2f1^{i2}$ females, the nurse cell nuclei appear dramatically reduced in size, which

suggests a defect in endoreplication or growth. Further experiment such as BrdU incorporation will be needed to confirm whether DNA replication is attenuated in *E2f1* mutant ovaries. In *Drosophila,* the endocycles in nurse cells are regulated by CycE/Cdk2 complex (Edgar and Orr-Weaver, 2001). As *cycE* is an important target of E2F transcriptional complex, the reduction of DNA content in *E2f1* mutant nurse cell nuclei might arise from the decrease of CycE level. Therefore, it would be interesting to know whether the expression levels of CycE is decreased in these nurse cells.

Another prominent feature of *E2f1* mutants is the altered pattern of *grk* expression during oogenesis. Consequently, *E2f1* mutant females lay eggs with eggshell phenotypes ranging from ventralized to dorsalized. The dorsovental patterning defects in *E2f1* mutant might be a result of the defective DNA replication in nurse cells, which is supported by the following considerations. First, nurse cells can synthesize most if not all *grk* transcripts during oogeneis, enough for proper establishment of dorsoventral and anteroposterior axes within oocyte (Caceres and Nilson, 2005). Thus, in the $E2f1^{07172}/E2f1^{i2}$ mutant, defects in some aspect of *grk* transcription, owing to lower DNA content, could affect localization of the mRNA. Second, in *spnB*, *spnD* and *okr* mutants (these genes encode proteins required for repair of double strand DNA breaks), a meiotic checkpoint is activated in response to unrepaired double-stranded DNA breaks (DSB). As a result, *grk* mRNA is mislocalized to the anterior region of the mutant oocyte, and Grk accumulation is greatly reduced (Ghabrial and Schupbach, 1999; Abdu et al., 2003). A similar mitotic checkpoint might be activated in E2f1 mutants in response to the DNA replication defects in nurse cells, which in turn affects the localized expression of *grk*.

Based on this hypothesis, elimination of the mitotic checkpoint should restore normal *grk* expression in *E2f1* mutants. Therefore, it will be interesting to test whether mutants of mitotic checkpoint genes, such as *mei-41* and *DmCHK*, will suppress the dorsoventral defects in E2f1 mutants (Brodsky et al., 2000; Garner et al., 2001; Hari et al., 1995; Sibon et al., 1999; Ghabrial and Schupbach, 1999; Abdu et al., 2002; Martinho et al., 1998; Matsuoka et al., 1998; Matsuoka et al., 2000).

Does E2f1 have a equivalent function in dorsoventral patterning as DP?

Mutation of DP, another E2F subunit, also affects dorsoventral polarity during oogenesis, but in a different way. Loss of *DP* function in germ line cells prevents Grk protein accumulation within the oocyte without altering the *grk* mRNA expression pattern. As a result, eggs from DP mutant mothers are ventralized (Myster et al., 2000). However, E2f1 mutant females lay eggs with eggshell phenotypes ranging from ventralized to dorsalized, as a result of the mild mislocalization of *grk* mRNA/protein at the anterior margin of the oocyte during midoogeneis. In addition to the differences in mutant phenotypes, mutations of *DP* and *E2f1* have different effects on Imp overexpression phenotype. Loss of E2f1 activity strongly enhances the dorsalized phenotype arising from Imp overexpression. In contrast, the deficiency covering the *DP* gene does not display any modification of the Imp overexpression phenotype. Therefore, *E2f1* and *DP* may not have equivalent function in dorsoventral polarity formation.

The differences in *E2f1* and *DP* mutant phenotypes might stem from the fact that DP can form heterodimer not only with E2f1, but also with E2f2, another E2f family

member in *Drosophila* (Frolov et al., 2001). E2f2 can bind to E2f1 binding sites, but has a distinct role from E2f1 in G1-S transition during mitosis (Sawado et al., 1998). Unlike E2f1/DP, which plays a positive role in transcriptional regulation of genes required for G1-S transition, E2f2/DP acts predominantly as a transcriptional repressor during *Drosophila* development (Sawado et al., 1998; Du, 2000). Removing E2f2 activity can repress the cell proliferation and DNA synthesis defects in *E2f1* mutants (Frolov et al., 2001), which supports a model that E2f1 and E2f2 act antagonistically during *Drosophila* development. Therefore, it will be interesting to know whether loss of E2f2 activity will modify the dorsoventral defects in *E2f1* mutants, and whether *E2f2* mutations alone can disrupt the dorsoventral polarity during oogenesis.

MATERIALS AND METHODS

Deficiency screen

The Exelixis deficiency collection (Chromosome II and III) used in this screen was obtained from Bloomington stock center. The cross scheme is shown in Fig 3.1. Two or three day old Imp overexpression females with or without Df/+ were sorted and put in apple juice vials with yeast paste to collect 0-24 hrs old eggs. As the dorsalized eggshell phenotype arising from Imp overexpression gets more severe as flies age, it is very essential to collect eggs from female flies that are less than a week old. Otherwise, it would be very hard to tell whether the Imp overexpression phenotype is enhanced in the presence of deficiency. For eggshell preparation, eggs were washed off from the apple juice vials with 0.1% Triton and filter through two layers of steel mesh to get rid of large pieces of agar, followed by flowing through a filter device to drain extra 0.1% Triton solution. The collected eggs were mounted in Hoyer's medium and visualized with Nikon microscope. The sqd^{ix50} flies were kindly provided by Trudi Schupbach. All the other fly stocks mentioned in this chapter were from the Bloomington stock center and Szeged stock center.

Cloning and transgenics

A full length *mub* cDNA (EST clone LD32520) was cloned into UASp vector to generate UASp-mub construct (Rorth, 1998). For another transgenic construct, UASp-mubGFP, part of the 5'UTR and coding sequence of *mub cDNA* were amplified by PCR

and fused to the N-terminal of GFP reading frame, which resides in a UASp vector. Transgenic stocks were generated by standard methods. The primers used in PCR are 5'-acaggtaccgtaggcaagccatcgaaaag-3', and 5'-acaggtaccgtgaatgggtgttttaacaattg-3'. Multiple independent lines of each transgene were obtained.

Western blot analysis

Protein samples were electrophoresed in a 10% SDS-polyacrymide gel and electroblotted to PVDF membrane. Proteins were detected by chemiluminescence (Western Light, Tropix). Primary antibodies were used at the following dilution: rat polyclonal anti-Mub at 1:500, mouse monoclonal anti-α-Tubulin at 1:20,000 (gift from Tim Stearns).

Immunodetection and in situ hybridization

For Grk staining, ovaries were dissected and stained as described (Macdonald et al., 1991; Macdonald and Struhl, 1986). Whereas immunostaining of the sponge bodies by Bru antibody was performed as described (Snee and Macdonald, 2004). Primary antibodies were used at the following dilutions: mouse anti-Gurken [1D12 from the Developmental Studies Hybridoma Bank (DSHB)], 1:10. rat anti-Bru (1:500). Secondary antibodies were labeled with Cy5 (Jackson Immunoresearch Laboratories) or Alexa Fluor 488 (Molecular Probes), 1:500. Stained ovaries were mounted in Vectashield medium (Vector Labs) and imaged with a Leica TCS-SP confocal microscope.

In situ hybridization was performed as described (Tautz and Pfeifle, 1989). Linearized plasmids containing the *grk* 3' UTR (p848 cut by BglII) were used as templates for synthesis of antisense RNA probes. The probes were labeled with Digoxigenin conjugated nucleotides (Roche Diagnostic GmbH).

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TABLES

Table 1. Deficiencies used in the screen. +: weak enhancement of Imp overexpression phenotype. ++: moderate enhancement of Imp overexpression phenotype**.** **+++:** strong enhancement of Imp overexpression phenotype. **-** : suppression of Imp overexpression phenotype (ventralized).

Deficiency	Break points	Modification
Df(2L)Exel6001	21A4;21B1	
Df(2L)Exel7002	21B4;21B7	
Df(2L)Exel8003	21D1;21D2	
Df(2L)Exel6002	21D2;21D3	
Df(2L)Exel7005	21D2;21D4	
Df(2L)Exel6003	21D3;21E3	
Df(2L)Exel6004	21E3;21F2	
Df(2L)Exel7006	21F2;21F4	
Df(2L)Exel8004	21F4;22A3	
Df(2L)Exel6005	22A3;22B1	
Df(2L)Exel7007	22B1;22B5	
Df(2L)Exel8005	22B2;22B8	
Df(2L)Exel6006	22B5;22D1	
Df(2L)Exel7008	22B8;22D1	
Df(2L)Exel6007	22D1;22E1	
Df(2L)Exel7010	22D4;22E1	
Df(2L)Exel7011	22E1;22F3	
Df(2L)Exel6008	22F3;23A3	
Df(2L)Exel6277	23A2;23B1	
Df(2L)Exel7014	23C4;23D1	
Df(2L)Exel7015	23D1;23E3	
Df(2L)Exel8008	23E3;23E5	
Df(2L)Exel7016	23E5;23F5	
Df(2L)Exel7018	24A1;24C2	

Df(2L)Exel6009	24C3;24C8
Df(2L)Exel8010	24C8;24D4
Df(2L)Exel6010	25A7;25B1
Df(2L)Exel9062	25B1;25B1
Df(2L)Exel8012	25B1;25B8
Df(2L)Exel7022	25B10;25C3
Df(2L)Exel7021	25B3;25B9
Df(2L)Exel8013	25B8;25B10
Df(2L)Exel6011	25C8;25D5
Df(2L)Exel6012	25D5;25E6
Df(2L)Exel7023	25E5;25F1
Df(2L)Exel6256	25E6;25F2
Df(2L)Exel8016	25E6;25F2
Df(2L)Exel6013	25F2;25F5
Df(2L)Exel6014	25F5;26A3
Df(2L)Exel7024	26A1;26A8
Df(2L)Exel6015	26B9;26C1
Df(2L)Exel6016	26C1;26D1
Df(2L)Exel9038	26C2;26C3
Df(2L)Exel7027	26F5;27B1
Df(2L)Exel7029	27C4;27D4
Df(2L)Exel8019	27E2;27E4
Df(2L)Exel6017	27E4;27F5
Df(2L)Exel7031	27F3;28A1
Df(2L)Exel6018	28B1;28C1
Df(2L)Exel9031	28B4;28C1
Df(2L)Exel7034	28E1;28F1
Df(2L)Exel8021	29C1;29D1
Df(2L)Exel7038	29C4;29D4
Df(2L)Exel7039	29D5;29F1
Df(2L)Exel7040	29F1;29F6
Df(2L)Exel6021	29F7;30A2
Df(2L)Exel7042	30B10;30C1
Df(2L)Exel8022	30B3;30B5
Df(2L)Exel9064	30B4;30B5
Df(2L)Exel6022	30B5;30B11
Df(2L)Exel9040	30C1;30C1
Df(2L)Exel6024	30C1;30C9

Df(2L)Exel6025	30C9;30E1	
Df(2L)Exel7043	30D1;30F1	
Df(2L)Exel8024	31A2;31B1	
Df(2L)Exel9032	31A3;31B1	
Df(2L)Exel7046	31C3;31D9	
Df(2L)Exel7048	31E3;31F5	
Df(2L)Exel8026	31F5;32B1	
Df(2L)Exel7049	32B1;32C1	
Df(2L)Exel6027	32D2;32D5	
Df(2L)Exel6028	32D5;32E4	
Df(2L)Exel6029	32E4;32F2	
Df(2L)Exel6030	33A2;33B3	
Df(2L)Exel6031	33B3;33C2	
Df(2L)Exel6032	33C2;33D4	
Df(2L)Exel6033	33E4;33F2	
Df(2L)Exel6034	33F2;34A1	
Df(2L)Exel8028	34A1;34A2	
Df(2L)Exel7055	34A2;34A7	
Df(2L)Exel7059	34D3;34E1	
Df(2L)Exel6035	35A3;35B2	
Df(2L)Exel6036	35B1;35B2	
Df(2L)Exel8033	35B1;35B8	
Df(2L)Exel8034	35C5;35D2	
Df(2L)Exel7063	35D2;35D4	+++
Df(2L)Exel6038	35D6;35E2	
Df(2L)Exel7066	36A1;36A12	
Df(2L)Exel6039	36A10;36B3	
Df(2L)Exel7067	36A12;36B2	
Df(2L)Exel8036	36B1;36C9	
Df(2L)Exel9044	36C10;36C11	
Df(2L)Exel7069	36C10;36D1	
Df(2L)Exel7068	36C7;36C10	
Df(2L)Exel7070	36D2;36E1	
Df(2L)Exel8038	36D3;36E3	+++
Df(2L)Exel9033	36E1;36E1	
Df(2L)Exel6041	36F5;37A2	
Df(2L)Exel7071	37A1;37A4	
Df(2L)Exel7072	37A2;37B6	

Df(2L)Exel7073	37B1;37B9	
Df(2L)Exel8039	37B8;37B11	
Df(2L)Exel6042	37B8;37C5	
Df(2L)Exel8040	37C1;37C5	
Df(2L)Exel6043	37C5;37D7	
Df(2L)Exel7075	37D2;37E1	
Df(2L)Exel8041	37D7;37F2	
Df(2L)Exel6044	37F2;38A4	
Df(2L)Exel6045	38A4;38A7	
Df(2L)Exel7077	38A7;38B2	
Df(2L)Exel6046	38C2;38C7	
Df(2L)Exel7078	38C7;38D4	
Df(2L)Exel7079	38E6;38F3	
Df(2L)Exel7080	38F3;39A2	
Df(2L)Exel6047	39A2;39B4	
Df(2L)Exel6048	39B4;39D1	
Df(2L)Exel7081	39D1;39E6	
Df(2L)Exel6049	40A5;40D3	
Df(2R)Exel6050	42C7;42D4	
Df(2R)Exel6051	42D4;42E4	
Df(2R)Exel6283	42E7;43A1	
Df(2R)Exel6052	43D1;43E5	
Df(2R)Exel6053	43D5;43E9	
Df(2R)Exel7092	43E5;43E12	
Df(2R)Exel6054	43E9;43E18	
Df(2R)Exel6055	43F1;44A4	
Df(2R)Exel7094	44A4;44B4	++
Df(2R)Exel6056	44A4;44C2	++
Df(2R)Exel7095	44B3;44C2	
Df(2R)Exel6057	44B9;44C4	
Df(2R)Exel6058	44C4;44D1	
Df(2R)Exel7096	44C6;44D3	
Df(2R)Exel8047	44D4;44D5	
Df(2R)Exel7098	44D5;44E3	
Df(2R)Exel8049	45F1;46A1	
Df(2R)Exel9016	46B1;46B2	
Df(2R)Exel6059	47C3;47D6	
Df(2R)Exel6060	47D6;47F8	

Df(2R)Exel6061	48F5;49A6
Df(2R)Exel7121	49B5;49C1
Df(2R)Exel8056	49D1;49E1
Df(2R)Exel7123	49D5;49E6
Df(2R)Exel6062	49E6;49F1
Df(2R)Exel8057	49F1;49F10
Df(2R)Exel7124	49F10;50A1
Df(2R)Exel7128	50C5;50C9
Df(2R)Exel7130	50D4;50E4
Df(2R)Exel7131	50E4;50F6
Df(2R)Exel8059	51A4;51B1
Df(2R)Exel6284	51B1;51C2
Df(2R)Exel7135	51E2;51E11
Df(2R)Exel9015	51F11;51F12
Df(2R)Exel9026	52A13;52A13
Df(2R)Exel6285	52A4;52B5
Df(2R)Exel7137	52B1;52C8
Df(2R)Exel7138	52D1;52D12
Df(2R)Exel7139	52D11;52E4
Df(2R)Exel9060	52E9;52F1
Df(2R)Exel6063	52F6;53C3
Df(2R)Exel7142	53A4;53C4
Df(2R)Exel6064	53C10;53D2
Df(2R)Exel7145	53C13;53D14
Df(2R)Exel7144	53C8;53C11
Df(2R)Exel6065	53D14;53F9
Df(2R)Exel6066	53F9;54B6
Df(2R)Exel7149	54C10;54D5
Df(2R)Exel7150	54E1;54E9
Df(2R)Exel7153	55B9;55C1
Df(2R)Exel7157	55E2;55E11
Df(2R)Exel7158	55E9;55F6
Df(2R)Exel6067	55F8;56A1
Df(2R)Exel6068	56A1;56B5
Df(2R)Exel6069	56B5;56C11
Df(2R)Exel7162	56F11;56F16
Df(2R)Exel7163	57A2;57A6
Df(2R)Exel7164	57A6;57A9

Df(2R)Exel6070	57A6;57B3
Df(2R)Exel6072	57B16;57D4
Df(2R)Exel6071	57B3;57B16
Df(2R)Exel7166	57B3;57B7
Df(2R)Exel6076	57D13;57F3
Df(2R)Exel6077	57F10;58A3
Df(2R)Exel7169	58A3;58B1
Df(2R)Exel7170	58B1;58C1
Df(2R)Exel6078	58B1;58D1
Df(2R)Exel7171	58C1;58D2
Df(2R)Exel7173	58D4;58E5
Df(2R)Exel7174	58E5;58F3
Df(2R)Exel6079	59A3;59B1
Df(2R)Exel7176	59B4;59C2
Df(2R)Exel7177	59C3;59D2
Df(2R)Exel7178	59D5;59D10
Df(2R)Exel7180	59E3;59F6
Df(2R)Exel7182	60A13;60A16
Df(2R)Exel7184	60B12;60C4
Df(2R)Exel6082	60C4;60C7
Df(2R)Exel9043	60C7;60C7
Df(2R)Exel7185	60C8;60D3
Df(3L)Exel6083	61A6;61B2
Df(3L)Exel6084	61B2;61C1
Df(3L)Exel9057	61C1;61C1
Df(3L)Exel6085	61C3;61C9
Df(3L)Exel6086	61C9;61E1
Df(3L)Exel6087	62A2;62A7
Df(3L)Exel6088	62B4;62B7
Df(3L)Exel6089	62D1;62D4
Df(3L)Exel6090	62E2;62E4
Df(3L)Exel6091	62E8;62F5
Df(3L)Exel6092	62F5;63A3
Df(3L)Exel6093	63C1;63D3
Df(3L)Exel6094	63D2;63E1
Df(3L)Exel6095	63E1;63E3
Df(3L)Exel6096	63E3;63E4
Df(3L)Exel6097	63E3;63F2

Df(3L)Exel6098	63F2;63F7
Df(3L)Exel6099	63F7;64A5
Df(3L)Exel9000	64A10;64B1
Df(3L)Exel8098	64A12;64B6
Df(3L)Exel9058	64B11;64B11
Df(3L)Exel6102	64B15;64C5
Df(3L)Exel9001	64B2;64B6
Df(3L)Exel6101	64B5;64B11
Df(3L)Exel9028	64B9;64B11
Df(3L)Exel7208	64B9;64B15
Df(3L)Exel6104	64C10;64D1
Df(3L)Exel6103	64C5;64C10
Df(3L)Exel6105	64D1;64D6
Df(3L)Exel6106	64D6;64E2
Df(3L)Exel6107	64E5;64F5
Df(3L)Exel7210	65A1;65A5
Df(3L)Exel8101	65A3;65A9
Df(3L)Exel6108	65A9;65A11
Df(3L)Exel6109	65C3;65D3
Df(3L)Exel6110	65E4;65E8
Df(3L)Exel8104	65F7;66A4
Df(3L)Exel6279	66A17;66B5
Df(3L)Exel9034	66A22;66B3
Df(3L)Exel6112	66B5;66C8
Df(3L)Exel6114	67B10;67C5
Df(3L)Exel9048	67D1;67D2
Df(3L)Exel6115	68E1;68F2
Df(3L)Exel6116	68F2;69A2
Df(3L)Exel6117	69D1;69E2
Df(3L)Exel6118	70A3;70A5
Df(3L)Exel9017	70B1;70B2
Df(3L)Exel6119	70B2;70C2
Df(3L)Exel6120	70D1;70D3
Df(3L)Exel6121	70D3;70D4
Df(3L)Exel6122	70D4;70D7
Df(3L)Exel6123	70D7;70E4
Df(3L)Exel6125	71A3;71B3
Df(3L)Exel6126	71A3;71B3

Df(3L)Exel6262	71B3;71C1	
Df(3L)Exel6127	72D1;72D8	
Df(3L)Exel6128	72D8;72D10	
Df(3L)Exel6129	72F1;73A2	
Df(3L)Exel6130	73B5;73D1	
Df(3L)Exel9002	73D1;73D1	
Df(3L)Exel9003	73D1;73D4	
Df(3L)Exel9004	73D1;73D5	
Df(3L)Exel7253	73D5;73E4	
Df(3L)Exel6131	74A1;74B2	
Df(3L)Exel6132	74B2;74D2	
Df(3L)Exel9006	75A4;75A6	
Df(3L)Exel6133	75B4;75B11	
Df(3L)Exel6134	75C7;75D4	
Df(3L)Exel9046	76A5;76A6	
Df(3L)Exel6135	76B11;76C4	
Df(3L)Exel9007	76B3;76B11	
Df(3L)Exel9008	76B3;76B11	
Df(3L)Exel9009	76B5;76B11	
Df(3L)Exel9011	76B8;76B11	
Df(3L)Exel9061	76C3;76C3	
Df(3L)Exel9045	76D1;76D2	
Df(3L)Exel6136	77B2;77C6	
Df(3L)Exel9065	78D5;78D5	
Df(3L)Exel9066	78D5;78D6	
Df(3L)Exel6137	78F4;79A4	++
Df(3L)Exel6138	79D3;79E3	
Df(3R)Exel6140	82A3;82A5	
Df(3R)Exel6141	82B3;82C4	
Df(3R)Exel6142	82D2;82D6	
Df(3R)Exel6143	82E4;82E8	
Df(3R)Exel9029	83A2;83A3	
Df(3R)Exel6144	83A6;83B6	
Df(3R)Exel7283	83B7;83C2	
Df(3R)Exel6145	83C1;83C4	
Df(3R)Exel7284	83C4;83D2	
Df(3R)Exel6146	84C8;84D9	
Df(3R)Exel6263	84E6;84E13	

Df(3R)Exel6148	84F12;85A2	
Df(3R)Exel6147	84F6;84F13	
Df(3R)Exel6149	85A2;85A5	
Df(3R)Exel8143	85A5;85B2	
Df(3R)Exel6150	85A5;85B6	
Df(3R)Exel6152	85C11;85D2	
Df(3R)Exel6151	85C3;85C11	
Df(3R)Exel9036	85D11;85D11	
Df(3R)Exel6153	85D21;85E1	
Df(3R)Exel6264	85D24;85E5	
Df(3R)Exel6154	85E9;85F1	
Df(3R)Exel6155	85F1;85F10	
Df(3R)Exel6265	85F10;85F16	
Df(3R)Exel6156	85F16;86B1	
Df(3R)Exel6157	86B1;86B2-3	
Df(3R)Exel6158	86C2;86C3	
Df(3R)Exel6159	86C3;86C7	
Df(3R)Exel7305	86C6;86C7	
Df(3R)Exel7306	86C7;86D7	
Df(3R)Exel8152	86D7;86D9	
Df(3R)Exel7308	86E1;86E8	
Df(3R)Exel6161	86E14;86E18	
Df(3R)Exel6276	86E14;86E18	
Df(3R)Exel7309	86E17;86F1	
Df(3R)Exel8154	86E17;86F6	
Df(3R)Exel9018	86E2;86E4	
Df(3R)Exel6160	86E4;86E14	
Df(3R)Exel8153	86E8;86E14	
Df(3R)Exel9019	86F6;86F7	
Df(3R)Exel7310	86F6;87A1	
Df(3R)Exel6163	87A1;87A4	
Df(3R)Exel6162	87A1;87B5	
Df(3R)Exel7312	87A4;87A7	
Df(3R)Exel8155	87A4;87A9	
Df(3R)Exel7313	87A9;87B5	++
Df(3R)Exel7317	87B10;87C3	
Df(3R)Exel7314	87B3;87B8	
Df(3R)Exel6164	87B5;87B10	-

Df(3R)Exel6165	87B5;87B10	-
Df(3L)Exel7315	87B8;87B9	
Df(3R)Exel7316	87B9;87B11	-
Df(3R)Exel6166	87C5;87C7	
Df(3R)Exel7318	87C7;87D5	
Df(3R)Exel6167	87D10;87E3	
Df(3R)Exel8157	87D8;87D10	
Df(3R)Exel8158	87E3;87E7	
Df(3R)Exel6168	87E3;87E8	
Df(3R)Exel7320	87E8;87F2	
Df(3R)Exel6170	87F10;87F14	
Df(3R)Exel6171	87F14;88A4	
Df(3R)Exel6288	87F14;88A4	
Df(3R)Exel6169	87F2;87F10	
Df(3R)Exel8159	88A4;88B1	
Df(3R)Exel7321	88A9;88B1	
Df(3R)Exel6267	88B1;88C2	
Df(3R)Exel8160	88C10;88D6	
Df(3R)Exel6275	88D1;88D7	
Df(3R)Exel6172	88D5;88D7	
Df(3R)Exel6173	88D7;88E1	
Df(3R)Exel6174	88F1;88F7	
Df(3R)Exel7326	88F7;89A5	
Df(3R)Exel6175	89A1;89A8	
Df(3R)Exel8162	89A5;89A8	
Df(3R)Exel7327	89A8;89B3	
Df(3R)Exel7328	89B1;89B9	
Df(3R)Exel7329	89B14;89B19	
Df(3R)Exel6269	89B17;89D2	
Df(3R)Exel7330	89B19;89D2	
Df(3R)Exel9055	89C7;89C7	
Df(3R)Exel8163	89D2;89D2	
Df(3R)Exel6270	89D2;89D8	
Df(3R)Exel6176	89E11;89F1	
Df(3R)Exel8165	89E8;89E11	
Df(3R)Exel6178	90E7;91A5	
Df(3R)Exel6179	91A5;91B5	
Df(3R)Exel9030	91B5;91B6	

Df(3R)Exel6180	91B5;91C5	
Df(3R)Exel6181	91C5;91D5	
Df(3R)Exel6182	91D5;91E4	
Df(3R)Exel6183	91E4;91F8	
Df(3R)Exel6184	92A5;92A11	
Df(3R)Exel6185	92E2;92F1	
Df(3R)Exel6272	93A7;93B13	
Df(3R)Exel6186	93E6;93F1	+++
Df(3R)Exel6187	93F1;93F8	
Df(3R)Exel6189	93F14;94A2	
Df(3R)Exel6188	93F8;93F14	
Df(3R)Exel6190	94A2;94A9	
Df(3R)Exel6191	94A9;94B2	+
Df(3R)Exel6192	94B11;94D3	
Df(3R)Exel6273	94B2;94B11	
Df(3R)Exel6193	94D3;94E4	
Df(3R)Exel6274	94E4;94E11	++
Df(3R)Exel6280	94E5;94E11	
Df(3R)Exel9012	94E9;94E13	
Df(3R)Exel6194	94F1;95A4	
Df(3R)Exel6195	95A4;95B1	
Df(3R)Exel9013	95B1;95B5	
Df(3R)Exel9014	95B1;95D1	
Df(3R)Exel6196	95C12;95D8	
Df(3R)Exel6197	95D8;95E5	
Df(3R)Exel6198	95E5;95F8	
Df(3R)Exel6199	95F8;96A2	
Df(3R)Exel8178	95F8;96A6	
Df(3R)Exel7357	96A2;96A13	
Df(3R)Exel6200	96A20;96B4	
Df(3R)Exel6201	96C2;96C4	
Df(3R)Exel9056	96C4;96C5	
Df(3R)Exel6202	96D1;96E2	
Df(3R)Exel6203	96E2;96E6	+
Df(3R)Exel6204	96F9;97A6	
Df(3R)Exel6205	97D12;97E1	
Df(3R)Exel6206	97E1;97E5	
Df(3R)Exel6208	97E5;97E11	

Df(3R)Exel6259	98C4;98D6
Df(3R)Exel6209	98D6;98E1
Df(3R)Exel6210	98E1;98F5
Df(3R)Exel6211	98F5;98F6
Df(3R)Exel6212	99A1;99A5
Df(3R)Exel9025	99B10;99B10
Df(3R)Exel6213	99C5;99D1
Df(3R)Exel6214	99D5;99E2
Df(3R)Exel6216	99F6;99F7
Df(3R)Exel6215	99F6;99F8
Df(3R)Exel7378	99F8;100A5
Df(3R)Exel9020	100A4;100A5
Df(3R)Exel8194	100A4;100A7
Df(3R)Exel6217	100A6;100A7
Df(3R)Exel7379	100B2;100B8
Df(3R)Exel6218	100B5;100C1
Df(3R)Exel6219	100C1;100C4

Table 2. Tested mutants within the region deleted by *Df(2R)Exel 6056* and *Df(2R)Exel 7094*.

genotype	corresponding genes	cytological location	allele class	phenotypic class	mutagen	modification of Imp overexpression phenotype
sna^{18}	*sna*	35D2	loss of function	lethal, recessive	EMS	moderate enhancement
P{SUPor-P}Tim17b2^{KG07430}	*Tim17b2*	35D2	N/A	viable, fertile	P-element	No
P{lacW}lace^{k05305}	*lace*	35D3-35D4	N/A	lethal, recessive	P-element	No
lace^{2}	*lace*	35D3-35D4	N/A	lethal, recessive	EMS	No
P{PZ}CycE^{05206}	*cyc E*	35D4	hypomorph	lethal, recessive	P-element	strong enhancement
CycE^{Ar95}	*cyc E*	35D4	amorph	lethal, recessive	EMS	strong enhancement
PBac{WH}CG15256^{f04709}	*CG15356*	35D3	N/A	N/A	P-element	No
P{SUPor-P}CG18477^{KG07704}	*CG18477*	35D3	N/A	N/A	P-element	No
PBac{WH}Or35a^{f02057}	*Or35a*	35D3	N/A	N/A	P-element	No

Table 3. Tested mutants within the genomic region deleted by *Df(3R)Exel 6186*.

genotype	corresponding gene	cytological location	allele class	phenotypic class	mutagen	modification of Imp overexpression phenotype
P{PZ}E2f^{07172}	E2f1	93E9--F1	amorph	lethal, recessive	P-element	strong enhancement
E2F^{i2}	E2f1	93E9--F1	N/A	female sterile, recessive	EMS	No

Table 4. Tested mutants within the region deleted by *Df(2L)Exel 8038*.

genotype	corresponding gene	cytological location	phenotypic class	mutagen	modification of Imp overexpression phenotype
FasIII ^{E25}	*Fas III*	36F2-4	Neuroanatomy defective, recessive, viable	Δ2-3	No
l(2)SH1622 ^{SH1622}	*Fas III*	36F2-4	lethal , recessive	P-element	No
P{PZ}RpS26 ^{04553}	*RpS26*	36F4	lethal , recessive	P-element	No
P{SUPor-P}RpS26 ^{KG00230}	*RpS27*	36F4	lethal , recessive	P-element	No
P{GT1}CG12750 ^{BG01636}	*CG12750*	36F5	viable, fertile	P-element	No
P{lacW}l(2)SH0931 ^{SH0931}	*CG12751*	36F5	lethal, recessive	P-element	No
P{lacW}l(2)SH1181 ^{SH1181}	*bsf*	36F5	lethal, recessive	P-element	No
P{EPgy2}Ntf-2r ^{EY05573}	*Ntf-2r*	36F5	viable, fertile	P-element	No
P{SUPor-P} Ntf-2r ^{KG00588}	*Ntf-2r*	36F5	viable, fertile	P-element	No
PBac{WH}CG31746 ^{f03296}	*CG31746*	36 E1	viable	P-element	No

Table 5. Tested deficiencies overlapping with *Df(2L)Exel 8038*.

Deficiencies	breakpoints	mutagen	modification of Imp overexpression phenotype
Df(2L)Exel8038	36D3;36E3	FLPase	strong enhancement
Df(2L)Exel7070	36D2;36E1	FLPase	No

Table 6. Tested mutants within the region deleted by *Df(3R)Exel 6274* and *Df(3R)Exel 9012*.

genotype	corresponding gene	cytological location	allele class	phenotypic class	mutagen	modification of Imp overexpression phenotype
P{XP}CG17083 ^{d00403}	*CG17083*	94 E9	N/A	N/A	P-element	No
pnt$^{\Delta 88}$	*pnt*	94E10-13	amorph	lethal, recessive	Δ2-3	No
P{PZ}pnt ^{07825}	*pnt*	94E10-13	N/A	lethal, recessive	P-element	No
pnt ^{2}	*pnt*	94E10-13	amorph	lethal, recessive	EMS	No
P{lacW}pnt ^{1277}	*pnt*	94E10-13	hypomorph	viable, (with pnt $^{\Delta 88}$) cell polarity defect	P-element	No
P{PZ}cnc ^{03921}	*cnc*	94E4-7	N/A	lethal, recessive	P-element	No
P{EPgy2}EY12544	*cdc16*	94 E9	N/A	viable, fertile	P-element	No
orb ^{dec}	*orb*	94 E9	amorph	female sterile	P-element	No

Table 7. Tested deficiencies overlapping with *Df(3R)Exel 6274* and *Df(3R)Exel 9012*.

Deficiencies	breakpoints	mutagen	modification of Imp overexpression phenotype
Df(3R)Exel6274	94E4;94E11	FLPase	strong enhancement
Df(3R)Exel6280	94E5;94E11	FLPase	no
Df(3R)Exel9012	94E9;94E13	FLPase	moderate enhancement
Df(3R)ED6103	94D3;94E9	FLPase	strong enhancement
Df(3R)BSC56	94E1-2;94F1-2	Δ2-3	strong enhancement
Df(3R)Exel6193	94D3;94E4	FLPase	No

Table 8. Modification of Imp overexpression phenotype by mutations within the region deleted by *Df(3L)Exel 6137*.

genotype	corresponding gene	cytological location	phenotypic class	mutagen	modification of Imp overexpression phenotype
P{PZ}mub ^{04093}	*mub*	79A2-3	lethal, recessive	P-element	moderate enhancement
P{GT1}mub$^{BG00074\ a}$	*mub*	79A2-3	viable, fertile	P-element	No
P{XP} d05482	*CG7458*	79A4	viable, fertile	P-element	No
P{EPgy2}EY07711	*DNA pol-η*	79A4	viable, fertile	P-element	No

a. There is no indication that this allele disrupts *mub* function.

Table 9. Modification of Imp overexpression phenotype by deficiencies overlapping with *Df(3L)Exel 6137*.

Deficiencies	breakpoints	mutagen	modification of Imp overexpression phenotype
Df(3L)Exel6137	78F4;79A4	FLPase	moderate enhancement
Df(3L)Pc-2q	78C5-6 ; 78E3-79A1	X-ray	No
Df(3L)ED4978	78D5 ;79A2	FLPase	moderate enhancement

Table 10. Tested mutants within the region deleted by *Df(2R)Exel 6056* and *Df(2R)Exel 7094*.

genotype	corresponding genes	cytological location	phenotypic class	mutagen	modification of Imp overexpression phenotype
P{lacW}l(2)SH0983	*CG12769*	44A4	lethal, recessive	P-element	No
P{lacW}l(2)SH1919	*lig*	44A4	lethal, recessive	P-element	moderate enhancement
P{SUPor-P}lig $^{KG08209\ a1}$	*lig*	44A4	N/A	P-element	No
PBac{RB}lig $^{e04268\ a2}$	*lig*	44A4	N/A	P-element	No
P{lacW}Vps28 ^{k16503}	*Vps28*	44A4	lethal, recessive	P-element	No
P{SUPor-P}slv ^{KG02506}	*slv*	44A4	viable, fertile	P-element	No
P{XP}sut1 ^{d07339}	*sut1*	44A4	N/A	P-element	No
P{SUPor-P}sut3 ^{KG10160}	*sut3*	44A4	viable, fertile	P-element	No
PBac{RB}CG8713 ^{e00867}	*CG8713*	44A4	N/A	P-element	No
P{EPgy2}CG8712 ^{EY07021}	*CG8712*	44A4	viable, fertile	P-element	No
P{SUPor-P}CG11210 ^{KG08546}	*CG11210*	44A4	N/A	P-element	No
P{EP}cul-4 ^{EP2518}	*cul-4*	44A4	N/A	P-element	No
P{SUPor-P}cul-4 ^{KG02900}	*cul-4*	44A4	N/A	P-element	No
P{lacW}l(2)s9998	*l(2)s9998*	44A4	lethal, recessive	P-element	No

a1, a2, there is no indication that these two alleles disrupt *lig* function.

Table 11. Tested deficiencies overlapping with *Df(2R)Exel 6056* and *Df(2R)Exel 7094*.

Deficiencies	**breakpoints**	**mutagen**	**modification of Imp overexpression phenotype**
Df(2R)Exel6056	44A4;44C2	FLPase	moderate enhancement
Df(2R)Exel7094	44A4;44B4	FLPase	moderate enhancement
Df(2R)Exel6057	44B9;44C4	FLPase	No
Df(2R)Exel7095	44B3;44C2	FLPase	No

Table 12. Tested deficiencies overlapping with *Df(2R)Exel 7096.*

Deficiencies	**breakpoints**	**mutagen**	**modification of *I*mp overexpression phenotype**
Df(2R)Exel7096	44C6;44D3	FLPase	moderate enhancement
Df(2R)Exel6058	44C4;44D1	FLPase	No

Table 13. Tested deficiencies within the region deleted by *Df(3R)Exel 7313*.

Deficiencies	breakpoints	mutagen	modification of Imp overexpression phenotype
Df(3R)Exel7313	87A9; 87B5	FLPase	moderate enhancement
Df(3R)Exel7314	87B3; 87B8	FLPase	No
Df(3R)Exel6162	87A1; 87B5	FLPase	No
Df (3R) E79	86F1-2; 87B9	EMS	"suppression" (Ventralized)
Df (3R) T-45	86F1-2; 87B5-6	N/A	No
Df(3R)ED5558	86F9; 87B11	FLPase	"suppression" (Ventralized)

Table 14. Tested mutants within the region deleted by *Df(3R)Exel 6203*.

genotype	corresponding gene	cytological location	allele class	phenotypic class	mutagen	modification of Imp overexpression phenotype
msi^{1}	*msi*	96E2-4	amorph	lethal, recessive	Δ2-3	weak enhancement
msi^{2}	*msi*	96E2-5	N/A	lethal, recessive	Δ2-3	very weak enhancement
$P\{GT1\}CG17383^{BG00794}$	*CG17383*	96 E6	N/A	viable, fertile	p-element	very weak enhancement

Table 15. Tested mutants within the region deleted by *Df(3R)Exel 6164* and *Df(3R)Exel 7316*.

genotype	corresponding gene	cytological location	allele class	phenotypic class	mutagen	modification of Imp overexpression phenotype
PBac{WH}CG17202 ^{f01979}	*CG17202*	87B9	N/A	N/A	P-element	No
Pp1-87B ^{1}	*Pp1-87B*	87B9-10	hypomorph	semi-lethal	EMS	No
P{lacW}Pp1-87B ^{j6E7}	*Pp1-87B*	87B9-10	N/A	lethal, recessive	P-element	No
PBac{PB}Aos1 ^{c06048}	*Aos1*	87B10	N/A	N/A	P-element	No
PBac{WH}Dip-C ^{f00706}	*Dip-C*	87B9	N/A	N/A	P-element	No
P{GT1}desat1 ^{BG00955}	*desat1*	87B10-11	N/A	viable, fertile	P-element	No
P{EPgy2}desat1 ^{EY07679}	*desat1*	87B10-11	N/A	viable, fertile	P-element	No

Table 16. Tested deficiencies overlapping with *Df(3R)Exel 6164* and *Df(3R)Exel 7316*.

overlapping deficiencies	breakpoints	mutagen	modification of Imp overexpression phenotype
Df (3R) E79	86F1-2; 87B9	EMS	“Suppression” (Ventralized)
Df (3R) T-45	86F1-2; 87B5-6	N/A	No
Df (3R) ED5558	86F9; 87B11	FLPase	“Suppression” (ventralized)
Df(3R)Exel6164	87B5; 87B10	FLPase	“Suppression”(ventralized)
Df(3R)Exel7316	87B9; 87B11	FLPase	“Suppression”(ventralized)
Df(3R)Exel6165	87B5; 87B10	FLPase	“Suppression”(ventralized)
Df(3R)Exel7317	87B10; 87C3	FLPase	No

Table 17. *Df(3R) 6164* dominantly " suppresses" the dorsalization phenotypes resulting from Imp overexpression.

	wild type	DA fused but expanded	DA fused or very close	moderately dorsalized	strongly dorsalized	n
W^{1118}	100%	0	0	0	0	78
UAS-Imp/+; MATII/+	80.80%	0%	0.90%	12.50%	5.80%	190
UAS-Imp/+; MATII/+; Df(3R)Exel6164/+	35.80%	26.30%	33.20%	3.20%	1.60%	208

Table 18. Complementation tests of *cyc E* mutants.

	cycE ^{05206}	*cycE*^{AR95}	*cycE* ^{k05007}	*cycE* ^{KG00239}	*cycE*^{KG07848}
cycE ^{05206}	L	L	L	L	V
cycE ^{AR95}		L	L	L	V
cycE ^{k05007}			L	L	V
cycE ^{KG00239}				L	V
cycE ^{KG07848}					V

V, viable. L, lethal.

cycE^{AR95} is a null allele. *cycE* ^{05206} is a hypomorphic allele. The allelic class of the other *cyc E* alleles is unknown yet.

FIGURES

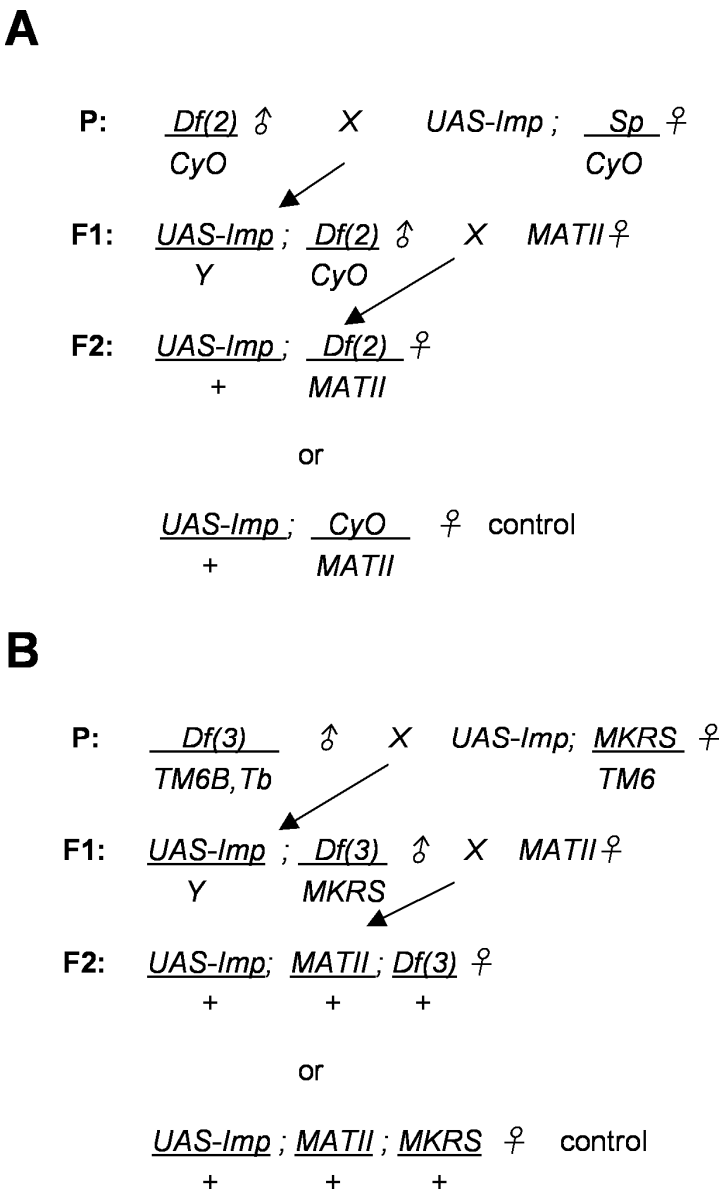


Figure 3.1 Cross scheme to test dominant modification of Exelixis deficiencies on Imp overexpression background. A. Cross scheme for chromosome II deficiencies. B. Cross scheme for chromosome III deficiencies. *MATII: matα4-GAL4VP16* driver on chromosome II.

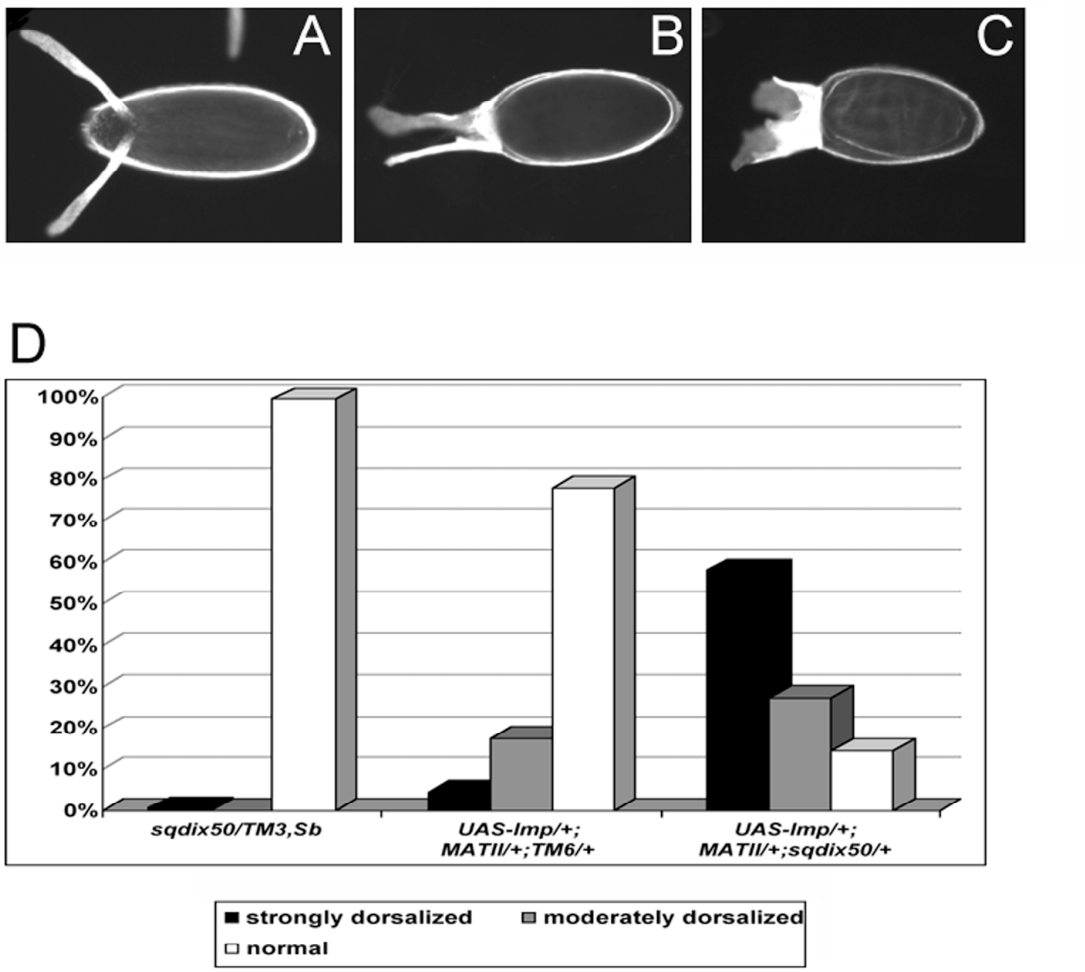


Figure 3.2 The Imp overexpression background for modification screen.

Imp overexpression in germ line cells results in various eggshell phenotypes ranging from wild type (A), moderately dorsalized (B), to strongly dorsalized (C). A strong allele of *sqd, sqd*^{ix50}, dominantly enhances the dorsalized eggshell phenotypes caused by Imp overexpression (D).

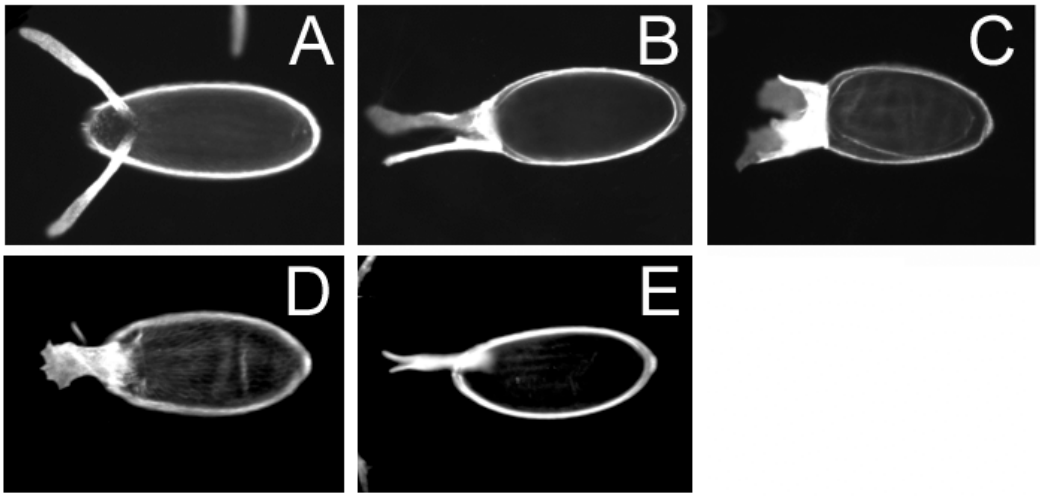
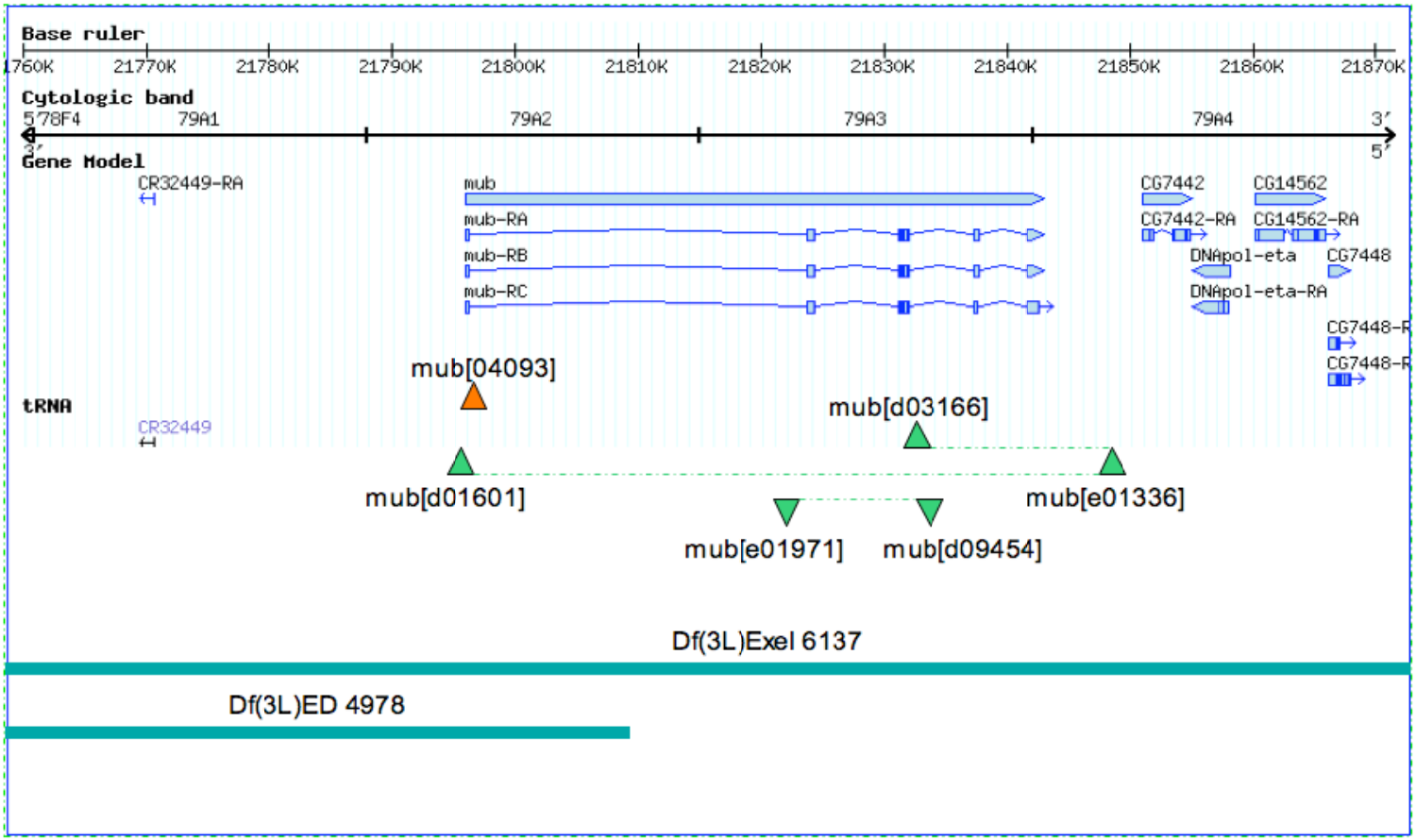


Figure 3.3 Defects of *UAS-Imp/+;MATII/+;Df(3R)Exel6164/+* derived eggshells. *UAS-Imp/+;MATII/+;Df(3R)Exel6164/+* females produce eggshells with a variety of dorsoventral phenotypes, including wild type eggshells (A); moderately dorsalized eggshells (B); strongly dorsalized eggshell, in which dorsal appendage material spreads and forms a ring at the anterior of the eggshell (C); eggshells with two dorsal appendages fused to form a single and wide one (D); and weakly ventralized eggshells with a single dorsal appendage (E).



gene span deficiency span mRNA deficiencies generated by Exelixis insertions

P{lacZ} insertion Exelixis insertions used to make *mub* deletions

(▽ transposons inserted in the sence strand. △ transposons inserted in the antisence strand.)

Figure 3.4 The genomic region of the *mub* gene.

This figure is adapted from the Flybase website (**http://flybase.bio.indiana.edu/cgibin/gbrowse/dmel/?ref=3L;start=21759284;stop=21895570**), showing the *mub* gene structure, transposon insertions and deficiencies that delete all or part of the *mub* gene.

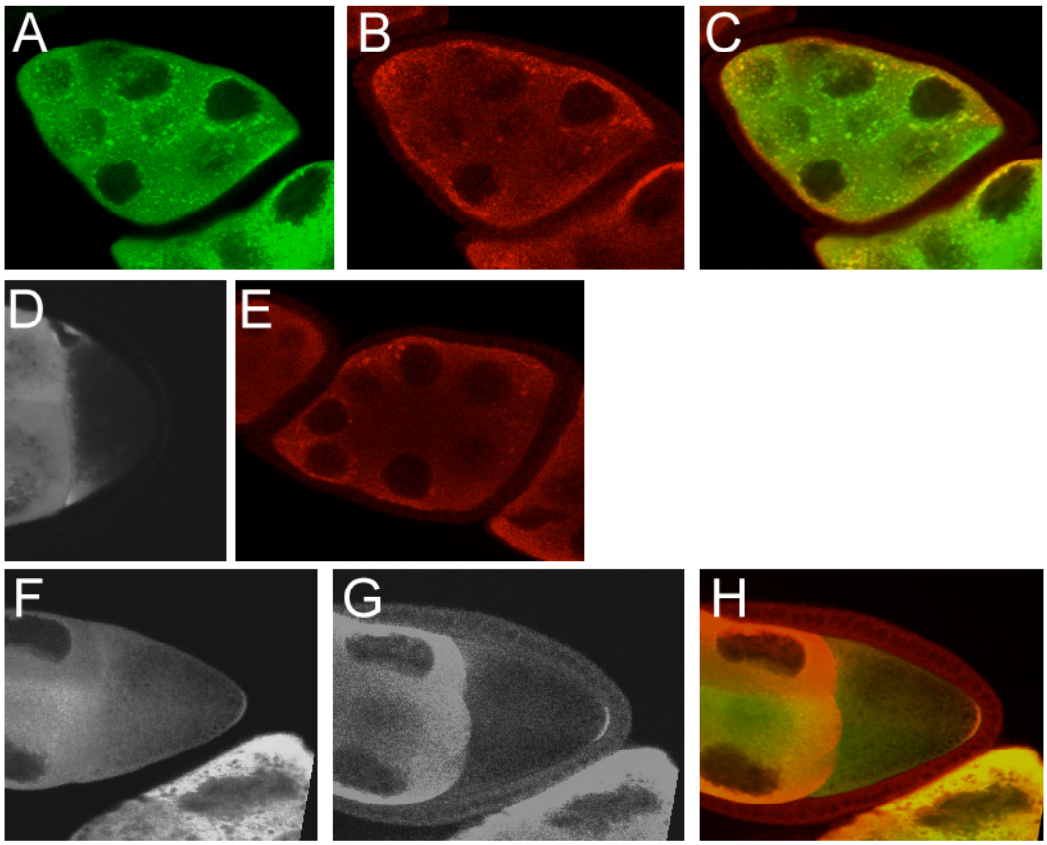


Figure 3.5 MubGFP distribution during oogenesis.

A-C, D, F-H, oocytes expressing MubGFP. E, wild type oocyte.

MubGFP forms puncta dispersed in the cytoplasm of nurse cell and oocyte (A), in which Bru, a sponge body component, is also detected (C). The distribution of Bru in a MubGFP expressing oocyte (B) is similar to that in a wild type oocyte (E). Therefore, MubGFP is enriched in sponge bodies. In addition, MubGFP is concentrated at the anterior margin of the oocyte during stage 8-9A (D). After stage 9A, MubGFP is detected at the posterior pole of the oocyte (F), colocalizing with Bru (H), which binds to *osk* mRNA specifically and concentrated at the posterior pole of the oocyte during midoogenesis (G).

Green: MubGFP; Red: Bru staining. C, H, merge of MubGFP and Bruno staining.

D,F mubGFP ; G: Bru staining.

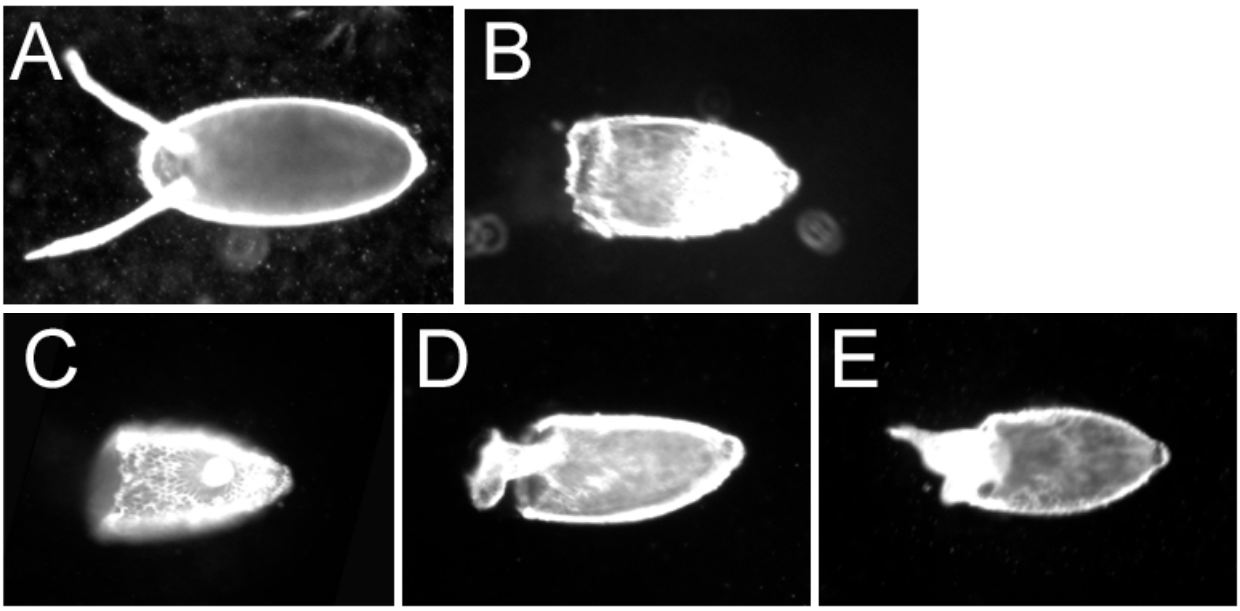


Figure 3.6 Overexpression of Mub in germ line cells causes eggshell defects.

Unlike eggshells from wild type mothers (A), most of the eggshells from Mub overexpression females are “cup like”, with the anterior ends failing to close. In addition, the dorsal appendages of these eggshells are either missing (B), or form a blob like structure positioned somewhere between the anterior and posterior ends of the eggshells (C), or form a single but slightly wide dorsal appendage (D), or are fused but widely spread out (E).

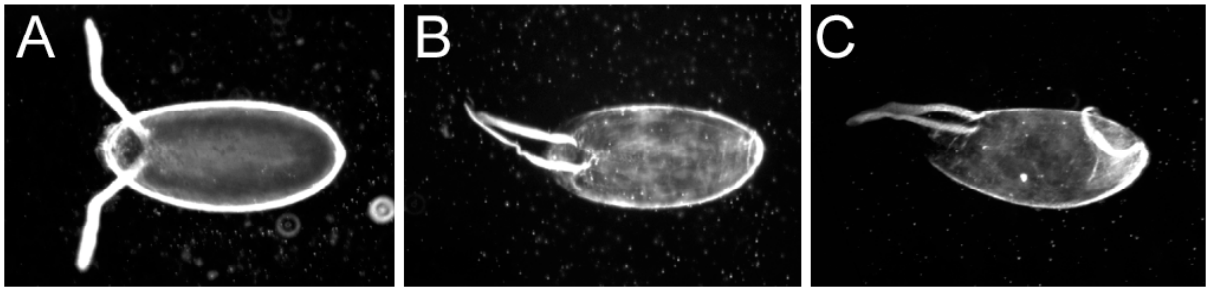


Figure 3.7 *cycE* mutations result in defective eggshells.

Unlike wild type eggshells (A), the eggshells derived from *cycE* ^{KG07848}*/Df(2L)osp29* females are thin and fragile, and their dorsal appendages are also thinner (B,C). Some of these eggshells have normal numbers of dorsal appendages (B), while others have additional dorsal appendage(s) located at random positions on the eggshells (C).

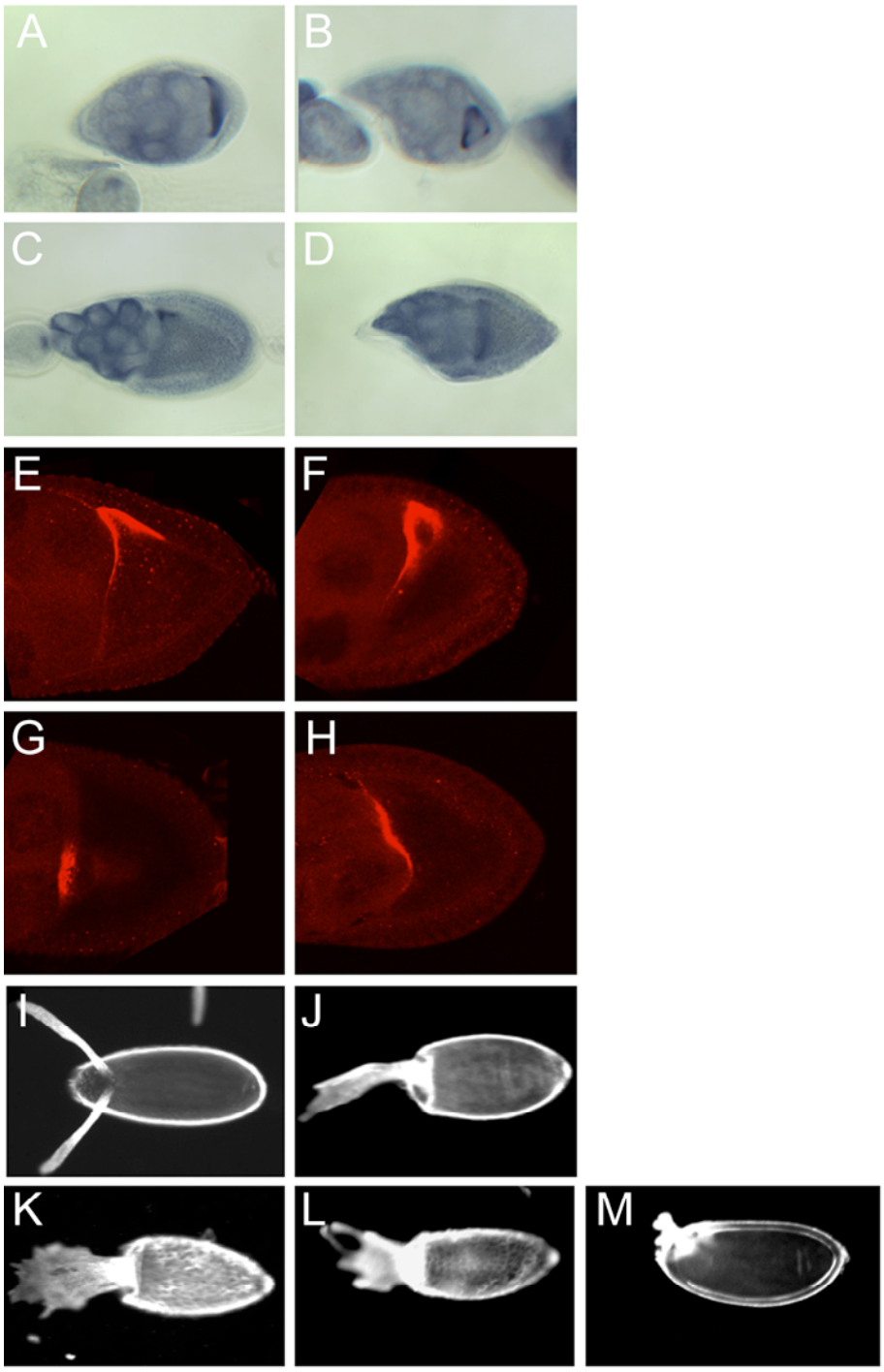

A
B
C
D
E
F
G
H
I
J
K
L
M

Figure 3.8 *E2f1* mutants have altered dorsoventral patterning and localized expression of *grk.*

A-D. Distribution of *grk* mRNA in wild type (A, C) and $E2f1^{07172}/E2f1^{i2}$ (B, D) eggchambers. In wild type eggchambers, *grk* mRNA is concentrated at the anterior margin of the oocyte at stage 7(A). However, *grk* transcripts are detected at both anterior and posterior poles of the $E2f1^{07172}/E2f1^{i2}$ oocyte (B). As oogenesis proceeds into stage 8-10, *grk* mRNA becomes restricted to the anterodorsal region of wild type oocyte (C). However, in some $E2f1^{07172}/E2f1^{i2}$ eggchambers, *grk* mRNA appears at both dorsal and ventral positions along the anterior margin of the oocyte (D).

E-H. Distribution of Grk protein in wild type (E, G) and $E2f1^{07172}/E2f1^{i2}$ (F,H) eggchambers at stage 9. In wild type eggchambers (E, G), Grk is normally localized at the anterodorsal region of the oocyte. In contrast, in $E2f1^{07172}/E2f1^{i2}$ eggchambers, Grk distibution is reduced dorsally and spreads slightly to the ventral position along the anterior margin of the oocyte (F, H). G, H, the dorsal surface view of Grk protein distribution. Note that the dorsal expansion of Grk is not shown in G and H.

I-J. Eggshells from wild type (I) and $E2f1^{07172}/E2f1^{i2}$ (J-M) mothers. The dorsal appendages are well separated in wild type (I). In contrast, *eggshells from* $E2f1^{07172}/E2f1^{i2}$ females display a variety of dorsoventral phenotypes, including eggshells with fused dorsal appendages (J, ventralized), eggshells with fused but slightly expanded dorsal appendages (K, between dorsalized and ventralized), and eggshells with fused but widely spread dorsal appendages (L, dorsalized). In addition, a small portion of eggshells from $E2f1^{07172}/E2f1^{i2}$ mothers have greatly degenerated dorsal appendages, which obscures the dorsoventral patterning of the eggshells (M).

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