**Src64 is required for ovarian ring canal morphogenesis during *Drosophila* oogenesis**

G. Steven Dodson¹, Douglas J. Guarnieri¹,² and Michael A. Simon¹,*

¹Department of Biological Sciences and ²Department of Genetics, Stanford University, Stanford, CA 94305, USA

*Author for correspondence (e-mail: msimon@leland.stanford.edu)

Accepted 18 May; published on WWW 9 July 1998

**SUMMARY**

The Src family of protein tyrosine kinases have been implicated as important regulators of cellular proliferation, differentiation and function. In order to understand further the role of Src family kinases, we have generated loss-of-function mutations in *Src64*, one of two Src family kinases known in *Drosophila melanogaster*. Animals with reduced *Src64* function develop normally and are fully viable. However, *Src64* female flies have reduced fertility, which is associated with the incomplete transfer of cytoplasm from nurse cells to the developing oocyte. Analysis of *Src64* egg chambers showed defects in the ring canals that interconnect the oocyte and its 15 associated nurse cells. *Src64* ring canals fail to accumulate the high levels of tyrosine phosphorylation that are normally present. Despite the reduced tyrosine phosphorylation, known ring canal components such as filamentous actin, a ring canal-specific product of the *hu-li tai shao* gene, and the *kelch* protein localize properly. However, *Src64* ring canals are reduced in size and frequently degenerate. These results indicate that *Src64* is required for the proper growth and stability of the ovarian ring canals.

Key words: Protein tyrosine kinase, src, *Src64*; Ring canal, *Drosophila*; Cytoskeleton, Egg chamber, Oogenesis

**INTRODUCTION**

The Src family of protein tyrosine kinases were first identified as transforming proteins encoded by oncogenic retroviruses (Collett and Erikson, 1978; Levinson et al., 1978). At present, nine distinct Src family kinases (SFKs) have been identified in vertebrates. The SFKs share a common domain structure consisting of an amino-terminal myristylation site, SH3 and SH2 domains, the protein tyrosine kinase catalytic domain and a carboxy-terminal regulatory region. Several of the vertebrate SFKs are expressed in specific hematopoietic lineages where their participation in receptor-mediated signaling is required for proper development and cellular function (for reviews of SFKs see Superti-Furga and Courtneidge, 1995; Brown and Cooper, 1996; Lowell and Soriano, 1996). The more broadly expressed SFKs (Src, Fyn and Yes) are activated in response to growth factors (such as PDGF, EGF, CSF-1) that signal through the activation of receptor tyrosine kinases (RTKs) (Raslton and Bishop, 1985; Osherov and Levitzki, 1994; Courtneidge et al., 1993). Consistent with a role in RTK signaling, the inhibition of SFK function blocks mitogenesis in response to these growth factors (Twanmley-Stein et al., 1993; Roche et al., 1995b). In addition to their involvement in receptor-mediated signaling, the inhibition of SFK function blocks the G2-M transition of the cell cycle in fibroblasts (Roche et al., 1995a).

Numerous studies suggest that SFKs also function to regulate the actin cytoskeleton. Transformation of fibroblasts with activated SFKs causes disruptions in the actin cytoskeleton (Boschek et al., 1981). These changes are associated with increased tyrosine phosphorylation of many cytoskeletal associated proteins. These include proteins involved in cell substrate adhesion (tensin, vinculin, talin, paxillin, FAK, β1 integrin, p130Cas, AFAP110), cell-cell adhesion (plakoglobin, β-catenin, p120Cas) and other proteins thought to regulate the actin cytoskeleton (p190 rhoGAP and cortactin) (Brown and Cooper, 1996). SFK participation in cytoskeletal regulation is also supported by studies of src-deficient mice. These mice suffer from osteopetrosis, a bone remodeling disorder caused by a failure of osteoclast function (Soriano et al., 1991; Lowe et al., 1993). Examination of the src− osteoclasts shows that they are deficient in the formation of ruffled borders and have defects in the underlying actin cytoskeleton (Boyce et al., 1992; Schwartzberg et al., 1997). Studies of fibroblasts derived from mice lacking Csk, a negative regulator of SFKs, have provided additional evidence for SFK involvement in actin cytoskeleton regulation. These cells have disrupted actin cytoskeletons and increased phosphorylation of p120, FAK, paxillin, tensin and cortactin. Furthermore, the removal of src activity suppresses the cytoskeletal defects of csk− cells and returns the phosphorylation of tensin and cortactin to normal levels (Thomas et al., 1995).

The developing egg chamber of *Drosophila* is useful for the study of cytoskeletal regulation. In *Drosophila*, oogenesis proceeds through fourteen discrete stages prior to the
fertilization of the egg. (for a review of *Drosophila* oogenesis see Spradling, 1993). At the initiation of oogenesis, a single germline-derived cystoblast cell under goes four rounds of mitotic divisions characterized by incomplete cytokinesis. As a result of the incomplete cytokinesis, these 16 germline cells are interconnected by a network of 15 cytoplasmic bridges called ring canals. One of the cells containing four ring canals becomes the developing oocyte while the 15 remaining cells differentiate as nurse cells. Surrounding each cluster of 16 cells is a sheath of somatically derived follicle cells. Early in oogenesis, the developing oocyte becomes transcriptionally inactive. Thus, most of the maternal products required for early embryogenesis are synthesized in the nurse cells and transported to the oocyte through the ring canals. Throughout most of oogenesis, the cytoplasmic transport from the nurse cells to the oocyte is gradual. At late stages (beginning at stage 11) of oogenesis, the nurse cells contract and rapidly transfer the remainder of their cytoplasmic contents to the oocyte (for a review on cytoplasmic transfer see Mahajan-Miklos and Cooley, 1994b). Following the completion of cytoplasmic transfer, the nurse cells degenerate.

The actin cytoskeletal rearrangements that occur during ring canal morphogenesis have been extensively studied (for a review of ring canals see Cooley and Robinson, 1996). Shortly after the arrest of the cleavage furrow, one or more unidentified phosphotyrosine-containing proteins localizes to the outer rim of the presumptive ring canal (Robinson et al., 1994). After the final mitotic divisions give rise to the 16 germ-cell cluster an inner rim forms at the ring canals. Initially, F-actin, a ringcanal-specific product (HTS-RC) of the *hu-li tai shao* (*hts*) gene and additional phosphotyrosine-containing protein(s) become localized to the inner rim of the ring canal (Robinson et al., 1994). The accumulation of F-actin is dependent on *hts* function since, in *hts* mutant egg chambers, the inner rim does not form at the majority of cytoplasmic bridges and only phosphotyrosine can be detected at most ring canals (Yue and Spradling, 1992; Robinson et al., 1994). Subsequent to the addition of F-actin, HTS-RC and phosphotyrosine protein(s), the *kelch* protein (Kelch) also becomes localized to the inner rim of the ring canal (Xue and Cooley, 1993). The function of Kelch is to maintain the compaction of the ring canal rim. In late stage *kelch* mutant egg chambers, F-actin diffuses into the inner lumen of the ring canals and partially blocks the transfer of nurse cell cytoplasm to the oocyte (Robinson et al., 1994; Tilney et al., 1996). In addition to these known components of the ring canal, the product of the *cheerio* gene is also required for proper ring canal formation. *Cheerio* ring canals are small and lack F-actin, HTS-RC and Kelch. Furthermore, fusions between the nurse cell and the oocyte are frequently observed in *cheerio* egg chambers indicating that the integrity of the plasma membrane has been compromised (Robinson et al., 1997). The *cheerio* gene has not been cloned so it is not known whether its product is a ring canal component.

Once the ring canals are established, they do not remain static. The rims of newly formed ring canals have diameters of 0.5-1 μm (Warn et al., 1985). By stage 11, at the onset of rapid cytoplasmic transfer from the nurse cells to the oocyte, the ring canals have attained their maximum size with a diameter of roughly 10 μm. EM studies have shown that the early phase of growth (prior to stage 5) is accompanied by the addition of new actin filaments to the ring canal. After stage 5, there is an increase in total F-actin at the ring canal, but it is unclear as to whether this increase results from the addition of new filaments or the thickening of existing filaments. However, during this developmental period, the filaments become organized into large bundles (Tilney et al., 1996).

We have generated mutations in *Src64*, one of two known *Drosophila* SFKs, and shown that *Src64* function is required during ring canal morphogenesis. *Src64* mutant egg chambers have dramatically decreased levels of phosphotyrosine at their ring canals. F-actin, HTS-RC and Kelch each localize properly to *Src64* mutant ring canals despite the reduction of phosphotyrosine levels. However, *Src64* ring canals fail to grow as large as wild-type ring canals. Furthermore, the mutant ring canals often degenerate prior to the onset of rapid cytoplasmic transfer. As a consequence, the cytoplasmic transfer from nurse cells to the oocyte is incomplete resulting in small eggs and reduced female fertility. Based on these observations, we propose that SRC64 is a key regulator of the growth and stability of the ring canals.

**MATERIALS AND METHODS**

**Drosophila stocks**

*All fly stocks were maintained under standard culture conditions. w1118 was used as wild type in these experiments. The BGT-T063 enhancer trap line, w1118; P{w[B]64C1-2}, was provided by Matthew Freeman and Gerry Rubin (University of California, Berkeley). The deficiency stock used in this study was w1118; Df(3L)10HTM3.Sb. For mobilization of the BGT-T063 P-element w1118; Ki, p, P[ry*, Del(2;2)3] flies were used as a transposase source.**

**Characterization of the *Src64* transcription unit**

The structure of the *Src64* transcription unit and the insertion site of the BGT-T063 P-element is based on direct nucleotide sequence comparison between genomic DNA and *Src64* cDNAs. Sequence of the genomic region containing the *Src64* protein-coding sequences was obtained by double-strand sequencing using directed primers based on the *Src64* cDNA sequence. Templates for the 5′ non-coding region of the *Src64* were obtained by sonication of a BGT-T063 rescue plasmid and insertion of the sonicated fragments into the vector M13mp10. Plasmid rescue of genomic DNA flanking P-element insertions has been described previously (Wilson et al., 1989). All sequencing was done by the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase kit (US Biochemicals). The third exon (28 bp) is present in the *Src64* cDNA and is unaccounted for in the genomic sequences from either the coding region or the 5′ region of *Src64*. It is presumed to lie in the large region that separates the 5′ end of the gene from the coding sequences.

**Generation and molecular characterization of *Src64* alleles**

The *Src64*117 and *Src64*117 alleles were isolated as imprecise excisions (Daniels et al., 1985) of the BGT-T063 P-element based on the loss of the white marker gene. DNA from 90 viable excision lines was used as templates for PCR reactions using the primers 5′-TAC-ACCGTTGAACGACTCGGACGCA-3′ and 5′-GCGTTGGTCTCGAGGGGAAAGGC-3′. Genomic Southern analysis showed that the deletions in *Src64*117 and *Src64*117 do not extend beyond the ClaI restriction site 5′ of the gene. The extent of the deletions into the gene has not been determined, but they remove most or all of the first two exons. Prior to the isolation of the *Src64*117 allele, EMS mutagenesis was used to generate lines in which the white gene in the BGT-T063 P-element insertion was mutated and gave a light yellow rather than a dark red eye color. One of these lines was then used to identify
potential local transpositions based on a darker red eye due to the presence of more than one P-element insert. Among these potential local transpositions, the Src64PI allele was identified due to its failure to complement the cytoplasmic transfer defect of the Src64PMT allele. The structure of Src64PI was determined by restriction digests and Southern blot analysis of rescue plasmids from the P-element insertions. The cytoplasmic transfer defect of Src64PI is reversible at high frequency (19/68). Genomic Southern analysis on Src64PI revertant lines indicates that mutant phenotypes are associated with the downstream P-element inserted within the Src64 transcription unit. For all the Src64 alleles, the cytoplasmic transfer defects are more severe when the alleles are in trans to a deficiency for Src64. For example, 55% of Src64PI stage 14 egg chambers show a clear cytoplasmic transfer defect, while 80% of Src64PI/Df3L(10H) stage 14 egg chambers are defective. These results suggest that none of the alleles are fully null for Src64 function.

Fertility analysis
An equal number of 2- to 5-day-old mutant or wild-type virgin females were crossed to wild-type males and their eggs collected on a molasses agar plate and counted at 24 hour intervals. The eggs were allowed to develop for another 28-30 hours and then examined under a dissecting microscope to quantitate the hatched versus unhatched eggs. These experiments were conducted at 25°C.

Generation of the anti-SRC64 serum
A rat anti-Src64 serum was generated to a GST-SRC64 fusion protein. A 4.2 kb NcoI-HindIII genomic DNA fragment was cloned into the pGEX-KG vector. The protein was expressed and purified from E. coli with glutathione affinity chromatography as previously described (Smith and Johnson, 1988) and injected into rats. The GST-SRC64 fusion protein contains the first 88 amino acids of the Src64 protein plus six random amino acids from the fourth intron of the Src64 gene.

Immunocytochemistry and imaging
Ovaries from 2- to 5-day-old females maintained in yeasted vials in the presence of wild-type male flies were dissected in phosphate-buffered saline (PBS). Fixation and antibody staining have been described previously (Cooley et al., 1992; Robinson et al., 1994). The anti-SRC64 serum was used at 1:500 dilution. The blots were then incubated with the anti-SRC64 serum at a 1:1000 dilution in TBST+0.5% w/v of dry milk. Blots were rinsed in TBST 4x 15 minutes prior to incubation with a peroxidase-coupled secondary antibody. The protein was detected using the ECL detection kit (Amersham).

Germline transformation
A 7 kb Apal-Sall genomic fragment of Src64, containing the Src64 protein-coding sequences, was cloned into the D277Maq vector provided by Daniel St Johnston. This vector is identical to the modified pCaTub67CMapolyA vector described previously (Micklem et al., 1997), except that the initiation ATG of Tub67c has been mutated to ATT. The cloning produced a transcriptional fusion between Tub67c and the untranslated region in the fourth exon of Src64 approximately 300 bp upstream of the Src64 initiation ATG.

RESULTS
We began our efforts to identify Src64 mutations by analyzing an enhancer trap line, BGT-T063, in which a P-element containing the white and lacZ genes was inserted near Src64 in the 64C1 polytene band of the third chromosome. Flies that are homozygous for the BGT-T063 P-element are viable and fertile. In order to map the location of the P-element insertion relative to the Src64 locus, we obtained genomic DNA flanking the P-element insertion site by plasmid rescue. Analysis of this DNA indicated that the P-element was inserted approximately 50 base pairs upstream of the 5' end of our longest Src64 cDNA (Fig. 1). Despite the proximity of the BGT-T063 P-element to the Src64 transcription unit, homozygous BGT-T063 embryos still express significant levels of Src64 protein (data not shown). However, flies in which the BGT-T063 P-element is placed in trans to a deficiency for the Src64 locus do occasionally show the mutant phenotypes described below thus indicating that the BGT-T063 insertion probably generates a very weak Src64 allele.

In order to identify strong Src64 mutations, we took advantage of the fact that transposase catalyzed P-element excision frequently results in the deletion of flanking DNA sequences. 200 lines of flies were established that contained chromosomes from which the BGT-T063 P-element had excised. Eight of the 200 excision chromosomes carried recessive lethal mutations. These eight lethal mutations either failed to map to the Src64 region or affected a previously identified gene near Src64 (data not shown). Since none of the homozygous lethal excisions appeared to affect Src64, we screened the homozygous viable excision chromosomes for lesions affecting the Src64 transcription unit. PCR primers were designed to amplify a 545 bp fragment from the non-
Src64 mutations alter ring canal morphogenesis

Incomplete cytoplasmic transfer is often indicative of defects in the actin cytoskeleton of the nurse cells. Two unique cytoskeletal features are particularly important for efficient transfer. The first is the formation prior to the rapid transfer phase (stage 10B) of actin cables that hold the nurse cell nuclei in place. In the absence of these actin cables, the nurse cell nuclei become lodged in the ring canals and block transfer (Cooley et al., 1992; Cant et al., 1994; Mahajan-Miklos and Cooley, 1994a; Guild et al., 1997). Staining of Src64 egg chambers with fluorescein-conjugated phalloidin to visualize filamentous actin at stage 10B showed that the actin cables were present (data not shown). The second important cytoskeletal structure of the nurse cells are the ring canals. In order to assess the role of SRC64 in ring canal morphogenesis,

Src64 mutations disrupt cytoplasmic transfer during oogenesis

Each of the Src64 alleles is viable both in trans to each other and to a deficiency for the Src64 region. However, females homozygous for any of the three alleles have reduced fertility (Table 1). Eggs laid by Src64 females hatch at reduced frequency when compared to wild type. In addition, Src64 females lay fewer eggs than wild type, which may suggest that the defective eggs are resorbed as has been previously observed for other female-sterile mutations (Spradling, 1993). In contrast, Src64 males are fully fertile. The loss of female fertility is associated with a defect in cytoplasmic transfer from the nurse cells to the developing oocyte. Unlike wild-type egg chambers, 55% of late stage Src64 egg chambers have nurse cell cytoplasm remaining at the anterior end of the oocyte (Fig. 3A,B). As a result, eggs from Src64 females range from 50%-100% of the length of eggs oviposited by wild-type females (Fig. 3C,D). Both the reduction of female fertility and the defect in cytoplasmic transfer can be reverted by excision of the downstream P-element present in the Src64 allele (data not shown). This indicates that these phenotypes are due to disruption of the Src64 gene.
early to mid-stage mutant egg chambers were stained with fluorescein phalloidin and with antibodies directed against phosphotyrosine, HTS-RC and Kelch. These experiments showed that the normal complement of 15 ring canals was present and that F-actin, HTS-RC and Kelch all localize properly at the ring canals (Fig. 4A-F). The intensity of the staining for these components did not differ appreciably from that observed for wild-type ring canals, but the ring canal morphology appeared abnormal (discussed below). In contrast, there was a significantly reduced level of anti-phosphotyrosine staining in Src64 egg chambers (Fig. 4G,H). This reduction was particularly dramatic at the ring canals, where only faint staining could generally be observed. However, mutant ring canals that maintained elevated anti-phosphotyrosine were observed occasionally. Reduced anti-phosphotyrosine staining was also observed in the cortical regions of the mutant nurse cells. These results indicate that Src64 function is required for the majority of phosphotyrosine accumulation at ring canals, but that the formation of the ring canals and the localization of known ring canal components does not depend on this accumulation.

During our analysis of ring canal morphogenesis, we observed that Src64 ring canals were smaller than their wild-type counterparts. This phenotype was particularly obvious in the later stages of oogenesis (Fig. 5A,B). This effect was quantitated by measuring the outer ring canal diameters during both mid and late stages (stages 5 and 10A) of oogenesis. In stage 5 wild-type egg chambers, the ring canals vary in size between 2.0 and 4.5 μm with an average size of 3.1 μm. In contrast, Src64PI ring canals vary in size from 1.0-3.5 μm with an average size 2.6 μm (Fig. 5D). The difference in ring canal size is more apparent at stage 10A (Fig. 5E). At this stage, wild-type ring canals vary between 6 and 14 μm with an average diameter of 9.5 μm. This represents a 3.1-fold increase in the average outer diameter of wild-type ring canals between stage 5 and stage 10A. In Src64PI egg chambers, there is only a 2.3-fold increase in ring canal diameter between stages 5 and 10A. Src64PI ring canals range from 3-10 μm with an average of 5.9 μm. These measurements indicate that ring canal growth is defective during both early and late phases of ring canal morphogenesis. In order to assess whether these smaller ring canals had other morphological abnormalities, F-actin-, HTS-RC- and Kelch-stained ring canals were examined at high magnification (Fig. 5C). The small ring canals appeared normal except for the presence of a slightly concave inner rim that is reminiscent of the inner rims of earlier stage wild-type ring canals.

We also observed that many (45%) stage 10A Src64PI egg chambers contained fewer than 15 ring canals. Src64Δ17 and Src64Δ19 egg chambers also showed this phenotype but to a lesser extent. This reduction in ring canal number was only observed in stage 9-10 egg chambers, indicating that some ring canals must degenerate during these later stages of oogenesis. One possible consequence of ring canal degeneration would be fusion between cells. Evidence for such fusions was sought by staining mutant egg chambers with fluorescein phalloidin to visualize the filamentous cortical actin at the nurse cell boundaries, and propidium iodide to visualize the nurse cell nuclei. At stages 9-10 approximately 70% of Src64PI mutant
egg chambers had fusions between nurse cells (Fig. 6B). Nurse cell fusion was also observed in Src64PI females at a lower frequency (Fig. 6C). In only one case did we observe a fusion between the nurse cells and the oocyte. Interestingly, in rare cases, we observed cytoplasmic F-actin-containing structures that appeared to be ring canals that had detached from the cortical actin. To show that these structures were ring canals, we stained the mutant egg chambers with fluorescein phalloidin, propidium iodide and anti-Kelch antibodies. This experiment confirmed that the structures within the cytoplasm contain ring canal components (Fig. 6D,E). The morphology of the detached ring canals was often aberrant. Their F-actin appeared more diffuse than normal which suggests that they may have been in the process of degeneration. These observations suggest that SRC64 is required to maintain the association between the ring canal and either the cortical actin or plasma membrane. Loss of attachment may lead to ring canal degeneration and nurse cell fusion.

SRC64 is enriched at ring canals

The altered morphogenesis and stability of Src64 ring canals and their reduced phosphotyrosine content suggested that SRC64 might be a ring canal component. To test this possibility, wild-type egg chambers were stained with anti-SRC64 antibodies. Specific staining was detected in the cortex of the nurse cells and appeared enriched at the ring canals in wild type, but not Src64A17, egg chambers (Fig. 7A-D). Co-staining for F-actin and SRC64 showed that SRC64 localization overlaps F-actin at the ring canal (Fig. 7E). These results are consistent with SRC64 being localized to the nurse cell and oocyte plasma membranes as well as at the inner rim of the ring canals.

Ring canal defects can be rescued by germline expression of Src64

As a final confirmation that the phenotypes that we observed are due to Src64 mutations, we rescued the Src64 defects by expression of wild-type SRC64 in the germline of Src64PI mutant females. A female germline-specific expression vector in which transcription is driven from the Tub67C promoter was used (Micklem et al., 1997; Matthews et al., 1989). The entire Src64-coding region was inserted into this vector and then introduced into the Drosophila genome by P-element-mediated germline transformation. We found that expression of Src64 in Src64PI egg chambers (Fig. 8C-D) partially rescued the ring canal growth defects. At stage 10A, the rescued ring canals averaged 8.5 μm in diameter, which is slightly smaller than in wild type (9.5 μm) but significantly larger than in the mutant (5.9 μm). Consistent with this rescue, elevated levels of anti-phosphotyrosine immunostaining were restored to the ring canals (Fig. 8A,B). Furthermore, the hatch rate of the eggs laid by Src64PI females carrying the Tub67c:Src64 transgene was increased from 5.5 to 68% and the rescued females laid morphologically normal eggs. These results confirm that the mutant phenotypes are due to the lack of Src64 function.

DISCUSSION

We have generated mutations in the Src64 gene by both imprecise excision and local transposition of a nearby P-element. Analysis of animals homozygous for either of the strongest two alleles indicates that Src64 protein expression is severely reduced. Despite reduced SRC64 expression, these flies are viable. Their only observable phenotype is partial loss of female fertility that is associated with an inability to completely transfer cytoplasm from nurse cells to the developing oocyte. Previous studies have shown that this cytoplasmic transfer is critically dependent on the integrity of the ring canals that interconnect the oocyte with its nurse cells. We therefore investigated the role of SRC64 during ring canal morphogenesis.

The most apparent defect in Src64 ring canals is their failure to grow at normal rates and to achieve wild-type size. The reduced size at stage 5 demonstrates that SRC64 function is
Src64 function during Drosophila oogenesis

required during the early phase of ring canal growth. Our measurements at stage 10A indicate that Src64 ring canals also grow slowly during the late phase of ring canal morphogenesis. In addition, the association between the ring canals and the plasma membrane/cortical actin is compromised during the later stages of oogenesis. These results suggest that SRC64 function may be required throughout ring canal development. However, we cannot exclude the possibility that the critical period for SRC64 function is early and that the failure of SRC64 to act early indirectly affects the late phase growth and stability of the ring canals.

Among the earliest detectable markers of developing ring canals is phosphotyrosine. The accumulation of phosphotyrosine-containing proteins is followed by the recruitment to the ring canals of F-actin and ring-canal-specific proteins such as HTS-RC and Kelch. We find that phosphotyrosine epitopes fail to accumulate in most Src64 ring canals. This indicates that the majority of tyrosine phosphorylation of ring canal proteins is in response to SRC64 function. Despite the low level of phosphotyrosine at Src64 ring canals, F-actin, HTS-RC and Kelch all localize properly. This suggests that the phosphorylation of components of the arrested cleavage furrow is not essential for the recruitment of any of the known ring canal components. Instead, the slow growth of Src64 ring canals and their observed detachment from the plasma membrane are consistent with a role for SRC64 in regulating reorganization of the ring canal rim and its attachment to the plasma membrane during ring canal growth.

SRC64 localization to the ring canals has important implications for the role of SRC64-dependent phosphorylation at the ring canal. One possibility is that SRC64 directly phosphorylates structural components of the ring canal in order to regulate ring canal morphogenesis. Alternatively, SRC64 may activate other protein tyrosine kinases that then phosphorylate inner rim components. The study of vertebrate SFKs has provided evidence for the activation of other protein tyrosine kinases by SFKs. For example, the transformation of cells by the activated v-src protein leads to the tyrosine phosphorylation and activation of focal adhesion kinase (FAK) (Kanner et al., 1990; Schaller et al., 1992; Guan and Shalloway, 1992). Studies have demonstrated a crucial role for FAK in regulating the actin cytoskeleton during cell-substratum adhesion (Illic et al., 1995). Vertebrate studies have also shown...
that members of the Tec family of tyrosine kinases, such as Bruton’s Tyrosine Kinase (BTK), can be activated by SFKs (Mahajan et al., 1995; Rawlings et al., 1996; Afar et al., 1996). Interestingly, we and others have recently shown that a Drosophila Tec family kinase, TEC29, is both localized to the ring canal in a SRC64-dependent manner and is required for proper ring canal growth (Guarnieri et al., 1998; Roulier et al., 1998).

Does Src64 have other functions during Drosophila development?

Previous studies have suggested that SRC64 may function at other times during Drosophila development. Src64 mRNA is broadly expressed throughout development with elevated levels detectable in the central nervous system (CNS) and developing visceral mesoderm of embryos and early pupae. High levels of Src64 were also detected in the photoreceptors of third instar eye imaginal discs (Simon et al., 1985). Since no Src64 alleles were available prior to this report, previous attempts to examine the role of SRC64 have relied on ectopic expression of a kinase inactive Src64 protein. In some cases, kinase inactive forms of SFKs have been shown to mimic the effects of disrupting the corresponding gene (Cooke et al.,

Fig. 6. Nurse cell fusion and ring canal degeneration in Src64 mutant egg chambers. (A-C) Stage 10 egg chambers stained with fluorescein phalloidin (shown in green) to visualize the nurse cell boundaries and propidium iodide (shown in red) to visualize the nurse cell nuclei. (A) Wild-type egg chamber; (B) Src64+/- egg chamber; (C) Src64-/- egg chamber. Nurse cell fusions in B and C are visible as cells with more than one nuclei surrounded by cortical actin. (D) Src64-/- egg chamber triple labeled with propidium iodide (shown in purple) fluorescein phalloidin (shown in green) and antibodies against Kelch (shown in red). The arrow indicates a detached ring canal within the cytoplasm of the fused nurse cells. The red cortical staining is non-specific background occasionally observed when working with the anti-Kelch antibodies (E) An enlargement of the detached ring canal in D. The scale bar in A is 50 μm. The scale bar in E is 12.5 μm. Anterior is to the left.

Fig. 7. SRC64 localization in egg chambers. (A) Wild-type stage 10 egg chamber stained with an antibody directed against the N-terminal domain of SRC64. SRC64 is detectable at the cortex of the nurse cells. In other sections, SRC64 can also be detected at the cortex of the oocyte. SRC64 was detected as early as stage 2. Prior to stage 2 the high background staining of the antibodies prevented unambiguous detection of the protein. (B) A Src64-/- stage 10 egg chamber stained with the anti-SRC64 antibodies. (C) Wild-type ring canals from an early stage egg chamber stained with fluorescein phalloidin to visualize the filamentous actin. (D) The same ring canals as in C stained with the anti-SRC64 antibodies. (E) A merged image of C and D with F-actin shown in green and Src64 in red. The resulting yellow ring canals indicate that SRC64 co-localizes with actin at the ring canal. The scale bar for A and B is 100 μm. The scale bar for C and D is 10 μm.
This difference in expression patterns may explain why the tissue where SRC41 expression may not overlap that of SRC64. preblastoderm embryos suggests that the female germline is a Y es causes an arrest of the cell cycle at the G2-M transition on cell cycle progression. However, inhibition of Src, Fyn and during G2 of the cell cycle does not have any dramatic effect (Lowell et al., 1996b). In fibroblasts, inhibition of Src alone marked increases the severity of the hck1994; Lowell et al., 1996a). Similarly, while the osteoclasts of in certain integrin-mediated cellular responses (Lowell et al., 1991; Stein et al., 1992; Appleby et al., 1992; Levin et al., 1993; Molina et al., 1992). In the case of SRC64, ubiquitous overexpression of the kinase inactive protein during embryogenesis led to defects in CNS development. During eye development, expression under sevenless transcriptional control of the kinase inactive SRC64 led to the loss of photoreceptors (Kussick et al., 1993). These results suggest that SRC64 might have an important role in CNS and photoreceptor development. However, we have been unable to detect any CNS or photoreceptor defects in Src64 animals. One possible explanation for this discrepancy is that the amount of SRC64 required during CNS and photoreceptor development is much lower than that required during oogenesis. Thus, the very low level of SRC64 present in the mutant animals might be sufficient for these processes. Alternatively, expression of the kinase inactive SRC64 may interfere with biochemical pathways in which SRC64 either does not normally participate or in which SRC64 function can be replaced by SRC41, the second Drosophila SFK (Takahashi et al., 1996).

The idea that some cellular functions can be performed by more than one SFK has been supported by previous studies. For example, while mice lacking either hck or fgr do not display any significant defects in myeloid development or function, the neutrophils of hck−fgr− mice show severe defects in certain integrin-mediated cellular responses (Lowell et al., 1994; Lowell et al., 1996a). Similarly, while the osteoclasts of hck− mice function normally, the removal of hck from src− mice markedly increases the severity of the src− osteoclast defect (Lowell et al., 1996b). In fibroblasts, inhibition of Src alone during G2 of the cell cycle does not have any dramatic effect on cell cycle progression. However, inhibition of Src, Fyn and Yes causes an arrest of the cell cycle at the G2-M transition (Roche et al., 1995a).

During embryonic and larval development, the expression of Src41 largely overlaps Src64 including high levels of expression in the CNS and visceral mesoderm (Takahashi et al., 1996). Thus SRC41 may be able to compensate for a lack of SRC64 in these tissues. Interestingly, the lack of maternally contributed Src41 in preblastoderm embryos suggests that the female germline is a tissue where SRC41 expression may not overlap that of SRC64. This difference in expression patterns may explain why the Src64 mutant phenotype is limited to defects in oogenesis. Ultimately, understanding the full range of SRC64 and SFK function during Drosophila development will require the identification of mutations in Src41 and any as yet unidentified Drosophila SFKs.

We thank Dr Matthew Freeman for his initial isolation of the BGT-T063 P-element, Dr Daniel St. Johnston for the germline expression vector and Carrie Steinberg for the injection of P-element constructs. We would like to thank members of our laboratory for their many helpful comments on this manuscript and work. We would also like to thank Dr Lynn Cooley and members of her laboratory, especially D. Robinson, for helpful discussions and the generous donation of antibodies. The early stages of this work were performed in the laboratories of Drs J. Michael Bishop and Gerald M. Rubin. This study was supported by a grant from the National Eye Institute (1R01EY9845) and an NSF National Young Investigator Award (MCB-9357009). During the course of this work, M. A. S. was a Searle Scholar, a Lucille P. Markey Scholar in Biomedical Sciences, and a Terman Fellow. G. S. D. and D. J. G. were supported by an NIH predoctoral training grant.

REFERENCES


