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Csk differentially regulates *Src64* during distinct morphological events in *Drosophila* germ cells

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The Src family protein tyrosine kinases (SFKs) are crucial regulators of cellular morphology. In *Drosophila,* Src64 controls complex morphological events that occur during oogenesis. Recent studies have identified key Src64-dependent mechanisms that regulate actin cytoskeletal dynamics during the growth of actin-rich ring canals, which act as intercellular bridges between germ cells. By contrast, the molecular mechanisms that regulate Src64 activity levels and potential roles for Src64 in additional morphological events in the ovary have not been defined. In this report, we demonstrate that regulation of Src64 by *Drosophila* C-terminal-Src Kinase (Csk) contributes to the packaging of germline cysts by overlying somatic follicle cells during egg chamber formation. These results uncover novel roles for both Csk and Src64 in a dynamic event that involves adhesion, communication between cell types and control of cell motility. Strikingly, Src64 and Csk function in the germline to control packaging, not in migrating follicle cells, suggesting novel functions for this signaling cassette in regulating dynamic adhesion. In contrast to the role played by Csk in the regulation of Src64 activity during packaging, Csk is dispensable for ring canal growth control, indicating that distinct mechanisms control Src64 activity during different morphological events.

KEY WORDS: *Src64*, *Csk*, Oogenesis, Ring canal, Follicle formation

INTRODUCTION

Drosophila oogenesis depends on precise temporal and spatial control of proliferation, cell fate determination and morphological changes in the germ cells and somatic cells that compose the ovary. Oogenesis begins at the anteriormost region of the ovary, called the germarium. A germline-derived cell undergoes four rounds of cell division that are characterized by incomplete cytokinesis, resulting in the formation of a germline cyst of 16 interconnected cells. Dynamic regulation of cytoskeletal events during this process allows for the transport of oocyte-specific components through the intercellular bridges from 15 support cells, or nurse cells, to one cell of the cyst, which becomes the oocyte. The intercellular bridges are stabilized by actin-rich structures called ring canals that expand in size throughout oogenesis to accommodate the increasing volume of materials transferred to the oocyte. About halfway through the germarium, the cyst is contacted by cellular processes extended by somatically derived cells, called follicle cells. Upon contact, the follicle cells proliferate, invade and encapsulate the cyst, forming a follicle cell-germ cell unit, or egg chamber. The newly formed egg chamber exits the germarium and develops through 14 stages, yielding a mature oocyte (King, 1970; Spradling, 1993; Robinson and Cooley, 1996).

Significant progress has been made in identifying the molecular mechanisms that regulate these events. One well-studied process is the regulation of ring canal morphogenesis by the Src family protein tyrosine kinase (SFK), Src64. In *Src64* mutants, ring canals form normally, but exhibit reduced growth and stability throughout development (Dodson et al., 1998). These defects are correlated with reductions in tyrosyl phosphorylation of ring canal components such as the protein tyrosine kinase Tec29 (Lu et al., 2004) and the actin-bundling protein Kelch (Kelso et al., 2002). Additionally, *Src64* mutants exhibit reduced rates of actin polymerization at the ring canal inner rim (Kelso et al., 2002), suggesting that the defects observed in *Src64* mutants are due to direct effects on actin cytoskeletal dynamics.

By contrast to our growing understanding of the downstream consequences of Src64 function at the ring canal, the mechanisms that regulate Src64 activity during oogenesis are unknown. Src64 is a member of a conserved group of SH3- and SH2-domain-containing protein tyrosine kinases (PTKs) that have been extensively studied in vertebrate systems, where nine SFK members phosphorylate dozens of target proteins. Activation of vertebrate SFKs occurs by two primary mechanisms. First, engagement of the SH3 or SH2 domain by an appropriate binding partner promotes the 'open' or active conformation of SFKs. Alternatively, dephosphorylation of a key tyrosine residue in the C-terminal tail of an individual SFK (Y547 in Src64) (Kussick and Cooper, 1992) disrupts its autoinhibited form, allowing activation to occur. The reverse reaction, phosphorylation of the same C-terminal tyrosine residue, promotes the 'closed', inactive conformation and is performed by C-terminal Src kinase (Csk) family members. The balance of negative regulation, largely by Csk, and positive regulation by a variety of signals determines SFK localization and activity levels, and subsequently the levels of tyrosyl phosphorylation on key target proteins (Brown and Cooper, 1996).

To understand Src64 regulation during oogenesis, we generated complete loss-of-function alleles of *Src64* ($Src64^{KO}$) or *Csk* and examined their effects on morphological events in the germ cells. As expected, $Src64^{KO}$ ovaries exhibited small ring canals as well as catastrophic ring canal attachment defects, demonstrating the increased phenotypic severity of ovaries lacking all *Src64* function compared with previously characterized hypomorphic mutations. By contrast, loss of *Csk* did not dramatically affect ring canal growth, suggesting that other methods of Src64 regulation are crucial during this process. Importantly, we found that regulation of Src64 by Csk

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was involved in the packaging of germline cysts by follicle cells, demonstrating that Src64 is differentially regulated during distinct events. Together, these results define key roles for regulation of Src64 by Csk during oogenesis and uncover novel functions for *Src64/Csk* in morphological events.

MATERIALS AND METHODS

Drosophila stocks

w;Src64^{KO} was generated using the Ends Out recombination technique (Gong and Golic, 2003) and the pEndsOut2 vector (kindly provided by J. Sekelsky). In *Src64^{KO}*, the *white* gene replaces 5550 bp of the *Src64* locus from –802 bp to +971 bp relative to the ATG and stop (Genome release 4.3 coordinates: 3L bp 4,574,517-4,580,068).

w;Src64$^{\Delta 17}$ (Dodson et al., 1998), and B1-93F (Ruohola et al., 1991) were described. Rescue experiments were performed using *w,P[OS64-WT]/+;Src64^{mut}/Src64^{mut}* where the wild-type *Src64* transgene is expressed under *oskar* promoter control (Ephrussi and Lehmann, 1992; Smith et al., 1992). *w;P[UASp-Src64^{Y547F}]* lines express constitutively activated *Src64* under UAS control. To express [Src64^{Y547F}], the transgenic line was crossed to *w^{1118};P[w[+mC]=GAL4::VP16-nos.UTR]MVD*. For clonal analysis, *w;Src64^{mut},FRT2A/GFP,FRT2A* larvae were heat shocked on days 3,4,5 after egg lay, and clonal phenotypes in adult ovaries assessed.

w;Csk^{j1D8} and *w;Csk^{S017909}* were from Bloomington and Szeged stock centers, respectively. *w;Csk$^{\Delta S13}$* is an imprecise excision of the P-element in *w;Csk^{S017909}*. *Csk* coding region mutations were generated by EMS mutagenesis and affect Csk protein as follows: *w;Csk^{Q156Stop}*, is a presumed null; *w;Csk^{D589N}* is a mutation in the catalytic aspartic acid, and *w;Csk^{E481K}* disrupts the kinase active site. Clonal phenotypes of *Csk* mutants were assessed using the FLP/DFS technique or by marking clones with GFP.

Egg lay and hatch rate analysis were as described (Dodson et al., 1998).

Generation of antibodies and immunoblotting

Affinity purified rabbit anti-Src64CT antibodies are directed against a peptide containing the terminal 13-amino acids of Src64. Rabbit anti-pY434 activation-specific Src64 antibodies were generated against the peptide RVIADDEpYCPKQG. Irrelevant antibodies were subtracted by passing whole serum over an unphosphorylated Y434-BSA column. Anti-pY434 antibodies were affinity purified on a BSA-pY434 column (Miermont et al., 2000). Immunoblot analysis was as described (Lu et al., 2004) using anti-Src64CT (1/1000) or anti-BicD antibodies (DSHB).

Immunocytochemistry and imaging

Ovary dissections and fixation were described (Guarnieri et al., 1998). Seven-day-old virgin females were placed with males on yeasted vials for 2 days before ovary isolation. Phalloidin (Molecular Probes) or propidium iodide staining was described (Guarnieri et al., 1998), as was immunofluorescence analysis with antibodies against Src64CT (1/8000), Hts (1/1000) (Xue and Cooley, 1993), Vasa (1/2000) (Hay et al., 1990), Orb (1/10, DSHB), Bazooka (1/500) (Wodarz et al., 1999), DE-Cadherin (1/25) (Oda et al., 1994), Armadillo (1/100, DSHB), Fas3 (1/1000, DSHB), Cut (1/10, DSHB), pY434 (1/100), β-Gal (1/1000, Promega). Images were collected using a Bio-Rad MRC1024 confocal laser scanning microscope and Lasersharp software. Image processing was done using Image J and Adobe Photoshop software.

RESULTS

Amorphic *Src64* mutation leads to severe ring canal growth defects

To assess ovary defects caused by the complete absence of *Src64*, we used the 'ends-out' homologous recombination technique (Gong and Golic, 2003) to delete the *Src64* coding region. In crosses between heterozygous flies, *Src64^{KO}* flies eclosed at the expected rate, indicating that zygotic *Src64* function is dispensable for viability. As expected, Src64 protein expression was undetectable by immunofluorescence or immunoblotting in *Src64^{KO}* ovaries (Fig. 1A,B). By contrast, the previously described allele *Src64$^{\Delta 17}$* (Dodson et al., 1998) had low levels of Src64 protein that were detectable only by immunoblotting (Fig. 1B). *Src64^{KO}* females displayed severe fertility defects, including dramatically reduced egg

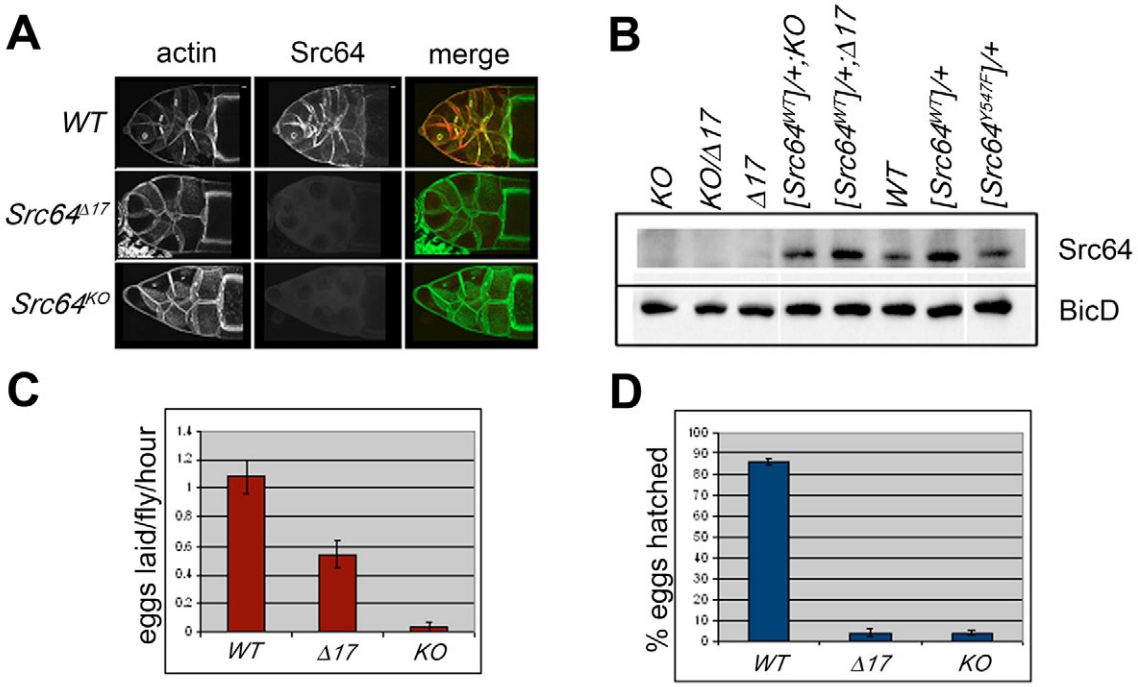

Fig. 1. *Src64^{KO}* fertility defects. (**A**) Confocal projections of stage 10 egg chambers stained with phalloidin (green) to visualize filamentous actin and anti-Src64 (red) to visualize Src64 protein. Genotypes are abbreviated. (**B**) Immunoblot of ovary extracts using anti-Src64 antibody or anti-BicD (loading control). (**C**) Egg lay rates for wild-type (WT) females versus *Src64$^{\Delta 17}$* or *Src64^{KO}* expressed as the number of eggs laid/fly/hour. (**D**) Hatch rates for WT versus *Src64* mutants. Scale bars: 10 μm.

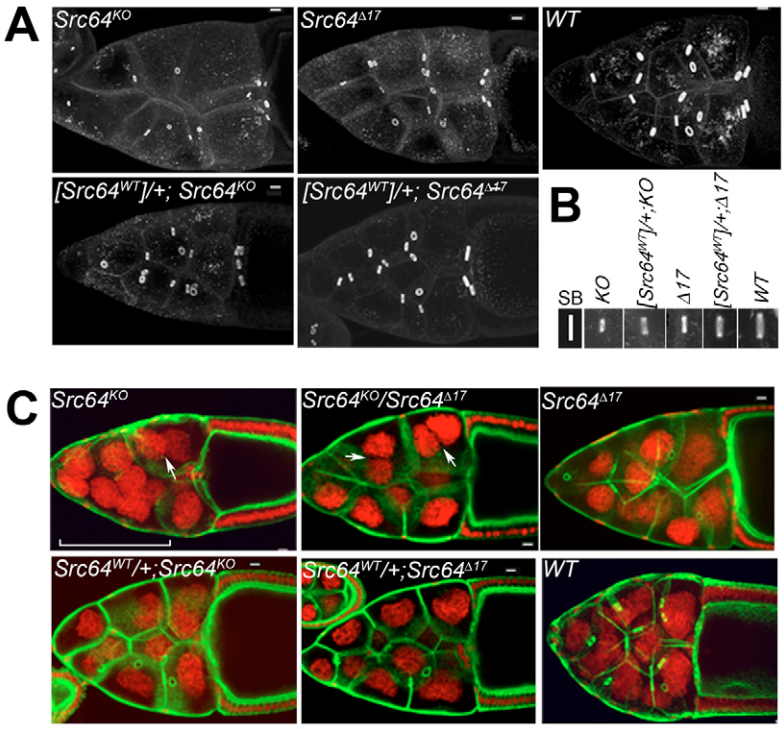

Fig. 2. *Src64* mutants exhibit ring canal morphology defects. (**A**) Confocal projections of stage 10 WT, *Src64* mutant (*Src64^{KO}*, *Src64^{Δ17}*), or rescued *Src64* mutant ([*Src64^{WT}*]/+;*Src64^{KO}* or [*Src64^{WT}*]/+;*Src64^{Δ17}*) egg chambers stained with anti-Hts-RC to visualize ring canals. (**B**) Average size ring canals labeled with anti-Hts-RC. (**C**) Partial confocal projections of stage 10 egg chambers labeled with phalloidin (green) to visualize filamentous actin and propidium iodide (red) to visualize DNA. Arrows and brackets indicate nurse cell fusion events. Scale bars: 10 μm.

lay and hatch rates (Fig. 1C,D). Egg lay rates in *Src64^{KO}* mutants were far more severe than those observed in *Src64^{Δ17}* hypomorphic mutants, suggesting that the small amount of Src64 protein found in *Src64^{Δ17}* mutants was sufficient to provide substantial Src64 function (Fig. 1C,D).

We next examined the effects of complete loss of Src64 function on ring canal morphology. Homozygous *Src64^{KO}* ring canals were smaller than both wild-type and *Src64^{Δ17}* ring canals (Fig. 2A,B, Table 1), and had undetectable levels of tyrosine phosphorylation (data not shown). Additionally, *Src64^{KO}* mutants exhibited severe defects in ring canal attachment to the cortical membrane relative to *Src64^{Δ17}* (Fig. 2C). *Src64^{KO}/Src64^{Δ17}* exhibited intermediate effects (Fig. 2C). *Src64* ring canal defects were rescued substantially by germline-specific expression of wild-type *Src64* (Fig. 1B, Fig. 2, Table 1), consistent with our previous reports (Dodson et al., 1998; Guarnieri et al., 1998). These results provide supporting evidence that Src64 function is crucial during ring canal morphogenesis and demonstrate that flies lacking all Src64 function exhibit more severe defects than flies retaining low expression levels.

Table 1. Ring canal size defects in *Src64* mutants

Genotype	Ring canal outer diameter (μm)*	Standard deviation (μm)	Statistical relationships†
w^{1118}	9.1	0.9	‡
w;Src64^{KO}	5.1	1.0	§, A, C
w;Src64^{Δ17}	6.8	1.2	§, B, c
w,[Src64^{WT}]/w;Src64^{KO}	7.1	0.6	a
w[Src64^{WT}]/w;Src64^{Δ17}	8.9	0.8	b
w,[Src64^{WT}]/w	8.7	0.8	¶
w;[UASpSrc64^{Y547F}]/ nosGal4VP16	9.0	1.1	¶

*All ring canals attached to the cortex were measured. In most cases, posterior ring canals were cortically attached, even in cases where drastic ring canal attachment defects occurred. The larger size of posterior ring canals may lead to an overestimation of ring canal sizes in mutants with ring canal attachment defects.
†Differences between ‡ and § were statistically significant, as determined by Student's *t*-test ($P<10^{-11}$). Differences between A and a or B and b or C and c were statistically significant ($P<10^{-5}$). Differences between ‡ and ¶ were not statistically significant ($P>0.05$). At least 85 ring canals were measured for each genotype.

Ring canal growth is normal in the absence of *Csk*

Significant progress has been made in identifying pathways downstream of *Src64* that regulate ring canal morphogenesis. The Tec29 protein tyrosine kinase and Kelch, an actin-bundling protein, localize to ring canals and are important Src64 targets (Xue and Cooley, 1993; Guarnieri et al., 1998; Kelso et al., 2002; Lu et al., 2004; Roulier et al., 1998). Whereas Tec29 and Kelch are regulated by localization, Src64 localizes uniformly to all germ-cell membranes, including the ring canals (Fig. 1A) (Dodson et al., 1998), suggesting that Src64 function is regulated predominantly at the level of activity rather than localization. Although proteins that activate *Drosophila* SFKs have not been identified, *Drosophila* Csk (Csk) has been shown to phosphorylate the regulatory C-terminal tyrosine of Src64 in vitro (Pedraza et al., 2004), demonstrating that this negative regulatory mechanism is conserved between flies and vertebrates.

If negative regulation of Src64 activity by Csk is important for regulation of ring canal growth, then three predictions can be made: (1) *Csk* mutants should exhibit ring canal growth defects; (2) constitutive activation of Src64 in the germ cells should mimic the effects of loss of *Csk*; and (3) reducing Csk levels in *Src64* mutants that retain a small amount of protein should lead to increased Src64 activity and suppression of defects.

To examine potential roles for Csk during ring canal morphogenesis, we generated *Csk* mutant germline clones (Chou and Perrimon, 1996). Homozygous mutation of previously described *Csk* alleles (Stewart et al., 2003; Read et al., 2004) and their derivatives did not affect germ cell development (Table 2, Table 4), but these alleles are not predicted to affect all *Csk* transcripts. We therefore examined three coding region mutations in *Csk* that eliminate Csk kinase activity and affect all *Csk* transcripts (see

Table 2. *Csk* mutation does not affect ring canal growth control

Genotype	Ring canal outer diameter (μm)	Standard deviation (μm)	Statistical relationships*
w,hsFlp;FRT82B/FRT82BovoD	10.3	0.8	†
w,hsFlp;FRT82Bcsk$^{\Delta 513}$/FRT82BovoD	10.7	0.7	‡
w,hsFlp;FRT82Bcsk ^{D589N}/FRT82BovoD	10.2	0.7	‡
w,hsFlp;FRT82Bcsk^{E481K}/FRT82BovoD	10.3	0.6	‡
w,hsFlp;FRT82Bcsk^{Q156Stop}/FRT82BovoD	10.7	0.7	‡
w;Src64$^{\Delta 17}$	6.5	0.4	§
w;Src64$^{\Delta 17}$,Csk/Src64$^{\Delta 17}$	7.0	0.5	¶
w;Src64$^{\Delta 17}$,Csk/Src64$^{\Delta 17}$,Csk	7.8	0.5	**

*Differences between † and ‡ were not statistically significant ($P>0.05$). Differences between § and ¶ ($P<0.05$) or ** ($P<10^{-5}$) were statistically significant, as determined by Student's *t*-test. At least 140 ring canals were measured for each genotype.

Materials and methods). Surprisingly, loss of *Csk* had no significant effect on average ring canal size relative to controls (Fig. 3A, Table 2). Similarly, normal-sized ring canals were observed in germ cells either expressing wild-type ([*Src64^{WT}*]) or highly activated ([*Src64^{Y547F}*]) (Kussick and Cooper, 1992) *Src64* transgenes in addition to endogenous *Src64* (Fig. 3B, Table 1). These results indicate that increasing Src64 activation in germ cells does not affect ring canal growth and that Csk is not the major regulator of Src64 during this process. However, we found that removal of one or two copies of *Csk* in *Src64$^{\Delta 17}$* mutants suppressed *Src64$^{\Delta 17}$* ring canal size defects (Table 2). These results suggest that Csk can regulate Src64 to control ring canal size when Src64 levels are limiting, but mechanisms that lead to Src64 activation normally are more crucial during this process.

Src64 mutant egg chambers have aberrant cell numbers

During our analysis of ring canal defects in *Src64* loss-of-function (*Src64^{LOF}*) mutants, we discovered egg chambers with too many or too few germ cells that were occasionally neighbors, and, on rare occasions, entirely fused ovarioles (Fig. 4, Table 3) (Djagaeva et al., 2005). By contrast to *Src64^{LOF}*, wild-type egg chambers always contained 16 germ cells (Table 3). The total germ cell number in defective *Src64^{LOF}* egg chambers was usually a multiple of 16, with the expected 15:1 ratio of nurse cells to oocytes (Fig. 4A,B). In cases where adjacent egg chambers were abnormal, the sum of the germ cells within both egg chambers often totaled a multiple of 16 and exhibited a 15:1 ratio of nurse cells to oocytes (Fig. 4C,D). The percentage of *Src64^{LOF}* egg chambers with aberrant cell numbers increased with age and temperature, and defective egg chambers were most prevalent when females were raised under optimal egg laying conditions. Germline expression of a wild-type *Src64* transgene in *Src64^{LOF}* backgrounds fully rescued the defects (Table 3), and egg chambers containing *Src64^{LOF}* germline clones also exhibited aberrant nurse cell numbers (Fig. 4F), demonstrating that *Src64* is required in the germline.

A possible explanation for the aberrant germ cell numbers in *Src64* mutant egg chambers is defective germ cell proliferation. The pattern of germ cell division during cyst formation dictates that wild-type oocytes always have four ring canals (Brown and King, 1964). If germ cells undergo an extra cell division, we expect oocytes to have five ring canals instead of four (Hawkins et al., 1996). Most *Src64^{KO}* egg chambers exhibited severe ring canal attachment defects, including floating ring canals and cortical membrane collapse, making it difficult to accurately score ring canal number in defective egg chambers (Fig. 4G). Therefore, oocyte ring canal number was scored in *Src64$^{\Delta 17}$* egg chambers that exhibited aberrant cell numbers. Every oocyte (n=52) in *Src64$^{\Delta 17}$* egg chambers with aberrant cell numbers had exactly four ring canals, indicating that extra cell division with incomplete cytokinesis could not explain the phenotype. Egg chambers with too many cells might also arise from extra cell divisions characterized by completed cytokinesis, leading to extra cells but fewer ring canals. Wild-type egg chambers have equal numbers (15 each) of nurse cell nuclei and ring canals. Similarly, equal numbers of nurse cell nuclei and ring canals were found in *Src64$^{\Delta 17}$* mutant egg chambers with aberrant germ cell numbers (n=10). Taken together, these results indicate that *Src64* mutant egg chambers with aberrant cell numbers do not arise due to changes in germ cell proliferation patterns.

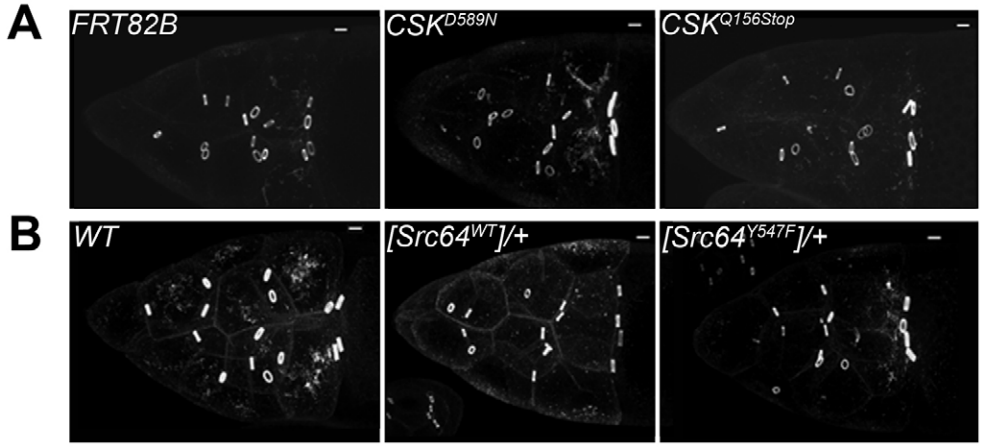

Fig. 3. Csk does not regulate ring canal growth. Confocal projections of stage 10 egg chambers stained with anti-Hts-RC to visualize ring canals are shown for (**A**) *FRT82B, Csk^{D589N}*, *Csk^{Q156Stop}*. (**B**) *WT*, overexpression of wild-type *Src64* ([*Src64^{WT}*]/+), expression of activated *Src64* ([*Src64^{Y547F}*]/+). For ring canals sizes, see Table 2. Scale bars: 10 μm.

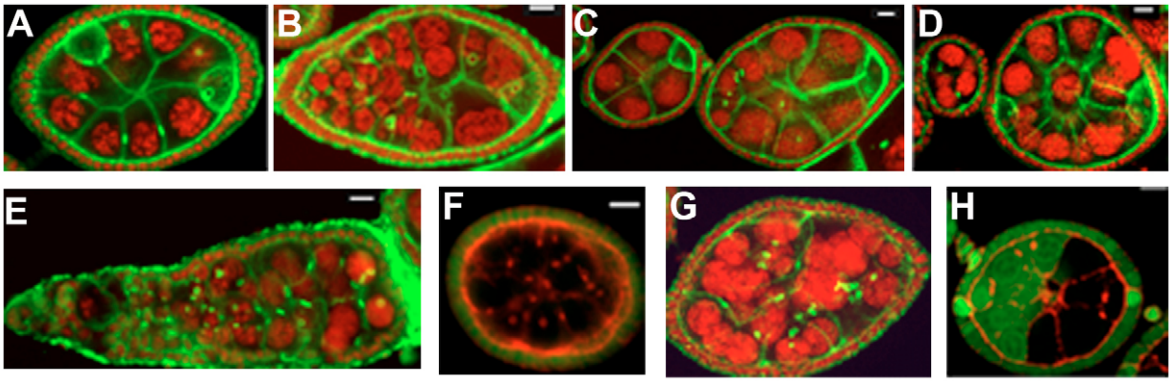

Fig. 4. *Src64^{LOF}* egg chambers exhibit packaging defects. (A-E,G) Partial confocal projections of *Src64^{LOF}* egg chambers labeled with phalloidin (green) to visualize filamentous actin and propidium iodide (red) to visualize DNA. (**A**) *Src64$^{\Delta 17}$* egg chamber containing 30 nurse cells (NC) + two oocytes (OO) (32 germ cells (gc) total). (**B**) *Src64$^{\Delta 17}$* egg chamber containing 60 NC + 4 OO (64 gc total). (**C**) *Src64^{KO}* egg chamber containing 22NC+1OO (right) next to egg chamber containing 8NC+1OO (32 gc total). (**D**) *Src64^{KO}* egg chamber containing 24NC+2OO (right) next to egg chamber containing 6NC (32 gc total). (**E**) *Src64^{KO}* ovariole. (**F**) *hsFlp/+;Src64$^{\Delta 17}$,FRT2A/GFP,FRT2A* egg chamber with too many germ cells labeled with phalloidin (red) and anti-GFP (green). *Src64* mutant cells lack GFP. (**G**) *Src64^{KO}* egg chamber containing too many cells as well as catastrophic membrane collapse. (**H**) *w,hsFlp/+;Src64$^{\Delta 17}$,FRT82B/Src64$^{\Delta 17}$,FRT82BGFP* egg chamber labeled with anti-GFP (green) and phalloidin (red). Half the cells in the egg chamber are labeled with GFP (green) and half are unlabeled, indicating that the cells come from independently derived germline cysts that were packaged together. Scale bars: 10 μm.

Src64 contributes to egg chamber formation

We next examined whether the observed phenotypes resulted from defects in the encapsulation or packaging of germline cysts by overlying follicle cells. We marked individual germline cysts by generating heatshock-induced GFP-labeled clones and aging the females for more than 8 days, thereby selecting for clones that were generated from a single germline stem cell division (King, 1970; Schupbach et al., 1978; Wieschaus and Szabad, 1979; Margolis and Spradling, 1995). This process ensures the genotypic equivalence of all cells in an individual cyst. We then looked for the presence of both GFP-labeled and unlabeled cells in *Src64^{LOF}* egg chambers with aberrant cell numbers, a phenotype that would suggest the packaging of cells derived from more than one cyst into a single egg chamber. Using this method, we found *Src64^{LOF}* egg chambers containing labeled and unlabeled cells (Fig. 4H), demonstrating that the aberrant egg chambers arose due to defective packaging.

To test whether increasing levels of *Src64* expression or activation affect packaging control, we compared the phenotypes observed upon germline expression of [*Src64^{WT}*] or highly activated ([*Src64^{Y547F}*]) *Src64* transgenes in a wild-type background. Germline overexpression of [Src64^{WT}] had no effect, whereas [Src64^{Y547F}] expression led to packaging defects (Fig. 5A, Table 3). These results indicate that inappropriate increases or decreases in Src64 activity levels cause packaging defects, suggesting that regulation of Src64 is involved in this dynamic event.

Src64 is regulated by Csk during packaging

To assess potential roles for Csk in regulating Src64 during packaging, we examined the phenotypes of *Csk* mutant ovaries. Whereas homozygous *Csk$^{\Delta S13}$* ovaries are indistinguishable from wild type, Csk germline mutants that lack kinase activity had effects on packaging that mimic expression of the highly activated [Src64^{Y547F}] (Fig. 5, Tables 3, 4). *Csk* egg chambers with aberrant cell numbers resulted from defective packaging, as oocytes (n=24) always had four ring canals, demonstrating that germ cell proliferation was normal, and GFP-labeled and unlabeled cells could be seen packaged together (Fig. 5C).

To test whether Csk regulates Src64 during packaging, we removed one copy of *Csk* in *Src64$^{\Delta 17}$* mutants, predicting that the activity of the small amount of Src64 present in *Src64$^{\Delta 17}$* mutants would increase upon removal of its negative regulator, leading to suppression of *Src64$^{\Delta 17}$* phenotypes. Consistent with this prediction, the high penetrance of packaging defects exhibited in *Src64$^{\Delta 17}$* mutants was suppressed upon removal of one copy of Csk in transheterozygous *Src64* mutants (*Src64$^{\Delta 17}$,Csk /Src64^{KO}*,+), and

Table 3. Quantitation of *Src64* packaging defects

Genotype	Percentage of ovarioles with packaging defects (%)	Standard deviation (%)	Number ovarioles scored (*n*)	Statistical relationships*
w^{1118}	0.4	1.0	1184	†
w;Src64^{KO}	14.2	5.5	864	‡, A
w;Src64^{KO}/Src64$^{\Delta 17}$	19.5	10.7	511	‡
w;Src64$^{\Delta 17}$	33.7	19.2	1362	‡, B
w,[Src64^{WT}]/w;Src64^{KO}	0.4	0.5	747	a
w,[Src64^{WT}]/w;Src64$^{\Delta 17}$	0.5	0.4	719	b
w,[Src64^{WT}]/w	0	0	217	§
w;[UASpSrc64^{Y547F}]/nosGal4VP16	6.1	4.9	487	‡

*Differences between † and ‡ were statistically significant, as determined by Student's *t*-test (P<0.001). Differences between A and a or B and b or were statistically significant (P<0.004). Differences between † and § were not statistically significant (P>0.05).

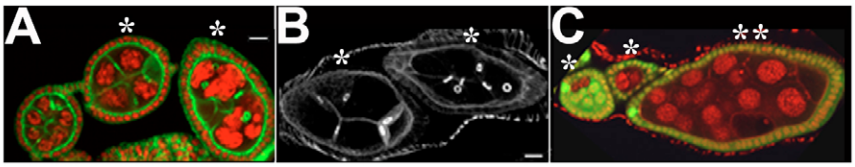

Fig. 5. Increasing Src64 activity leads to packaging defects. Partial confocal projections of egg chambers containing too few (*) or too many (**) cells. (**A**) [Src64^{Y547F}]-expressing egg chambers labeled with phalloidin (green) to visualize filamentous actin and propidium iodide (red) to visualize DNA. (**B**) *Csk^{D589N}* egg chambers labeled with phalloidin. (**C**) *Csk^{E481K}* mutant tissue is marked by the absence of GFP (green). Nuclei are labeled with propidium iodide (red). The youngest egg chamber includes labeled and unlabeled cells; thus *Csk^{E481K}* germline mutation leads to packaging defects. Scale bars: 10 μm.

dramatically suppressed in *Src64$^{\Delta 17}$* homozygotes (*Src64$^{\Delta 17}$,Csk/Src64$^{\Delta 17}$,+*) (Table 4). By contrast, the penetrance of packaging defects in *Src64^{KO}* homozygotes was unaffected by removal of one copy of *Csk* (Table 4). These results support a model in which Src64 activity is regulated by Csk during packaging, and imply that packaging defects observed in Csk germline clones are a consequence of unregulated Src64 activation.

Early germ cell differentiation and morphology are normal in *Src64* mutants

We next examined the possibility that defects in germ cell identity or germ cell-germ cell adhesion might allow invading follicle cells to separate cysts inappropriately. *Src64* mutant germ cells express Vasa (Fig. 6A), suggesting that germ cell differentiation is normal (Hay et al., 1990). Additionally, Orb and Bicaudal D (BicD), which mark all germ cells at early stages and become concentrated in the oocyte by stage 1 (Lantz et al., 1994; Suter and Steward, 1991), were expressed and localized normally in mispackaged *Src64* egg chambers (Fig. 6B), suggesting that oocytes are properly specified.

Although oocyte specification occurred normally in most mispackaged egg chambers, some *Src64* egg chambers exhibited 16 polyploid nuclei and lacked oocytes. In most cases, oocyte absence was correlated with cortical membrane collapse, and sometimes leaking of Orb protein could be observed (Fig. 6B). Egg chambers containing 16 polyploid cells in which the cortical membrane appeared intact might arise due to defects in transport of oocyte-specific materials early in development (Djagaeva et al., 2005); however, these egg chambers are extremely rare and do not represent a major *Src64^{LOF}* phenotype. Our results suggest that oocytes were generally specified normally in *Src64^{KO}* mutants, but defects in ring canal attachment led to the release of oocyte-specific components and subsequent conversion to a nurse cell fate.

The integrity of individual germline cysts must be maintained by adhesion between the germ cells for proper packaging to occur. In cases where germ cell-germ cell adhesion is compromised, follicle cells can invade inappropriately, separating nurse cells (Abdelilah-Seyfried et al., 2003; Goode and Perrimon, 1997; Peifer et al., 1993). If *Src64* packaging defects are due to defects in germ cell-germ cell adhesion, we predict that germ cell shape and localization of key adhesion molecules will be altered in cysts lacking *Src64* (Peifer et al., 1993). However, *Src64$^{\Delta 17}$* nurse cells exhibited normal morphology, including appropriate size, straight membranes and localization of germ cell-germ cell adhesion molecules such as Bazooka (Baz) and Armadillo (Arm) (Peifer et al., 1993; Oda et al., 1997; Goode and Perrimon, 1997; Godt and Tepass, 1998; Huynh et al., 2001; Cox et al., 2001) (Fig. 7D,E). *Src64^{KO}* germ cells were indistinguishable from wild-type or *Src64$^{\Delta 17}$* germ cells in terms of size and localization of adhesion molecules in the germarium. However, in many cases nurse cell morphology was subsequently affected by detachment of ring canals from the cortical membrane as early as stage 1 (data not shown). These changes in nurse cell morphology are unlikely to be the primary cause of *Src64^{LOF}* packaging defects, because *Src64$^{\Delta 17}$* mutants exhibited dramatic packaging defects without changes in germ cell morphology.

Src64 mutants exhibit normal follicle cell proliferation, differentiation and polarity

Defects in follicle cell proliferation, differentiation or morphology frequently lead to dramatic packaging defects (Horne-Badovinac and Bilder, 2005). Follicle cells with diminished proliferation rates fail to produce sufficient cell numbers to surround each germline cyst, resulting in gaps in the somatic epithelium and packaging defects (Jackson and Blochlinger, 1997; Zhang and Kalderon, 2000; Oh and Steward, 2001; Besse et al., 2002). Conversely, maintenance of follicle cells in an extended proliferative state prevents the

Table 4. Src64 is regulated by Csk during packaging

Genotype	Percentage of ovarioles with packaging defects (%)	Standard deviation (%)	Number ovarioles scored (*n*)	Statistical relationships*
w,hsFlp;FRT82B/FRT82BovoD	3.3	2.3	354	†
w,hsFlp;FRT82Bcsk$^{\Delta 513}$/FRT82BovoD	0.3	0.6	343	
w,hsFlp;FRT82Bcsk^{D589N}/FRT82BovoD	71.4	8.3	112	‡
w,hsFlp;FRT82Bcsk^{E481K}/FRT82BovoD	54.8	27.4	244	‡
w,hsFlp;FRT82Bcsk^{Q156Stop}/FRT82BovoD	24.4	10.0	372	‡
w;Src64^{KO}	26.0	9.8	222	
w;Src64^{KO},Csk/Src64^{KO}	33.6	19.6	549	
w;Src64$^{\Delta 17}$/Src64^{KO}	33.6	1.5	213	
w;Src64$^{\Delta 17}$,Csk/Src64^{KO}	21.3	9.0	503	
w;Src64$^{\Delta 17}$	42.0	1.1	311	§
w;Src64$^{\Delta 17}$,Csk/Src64$^{\Delta 17}$	8.7	2.7	607	¶

*Differences between † and ‡ were statistically significant, as determined by Student's *t*-test ($P<0.002$). Differences between § and ¶ were statistically significant ($P<10^{-4}$).

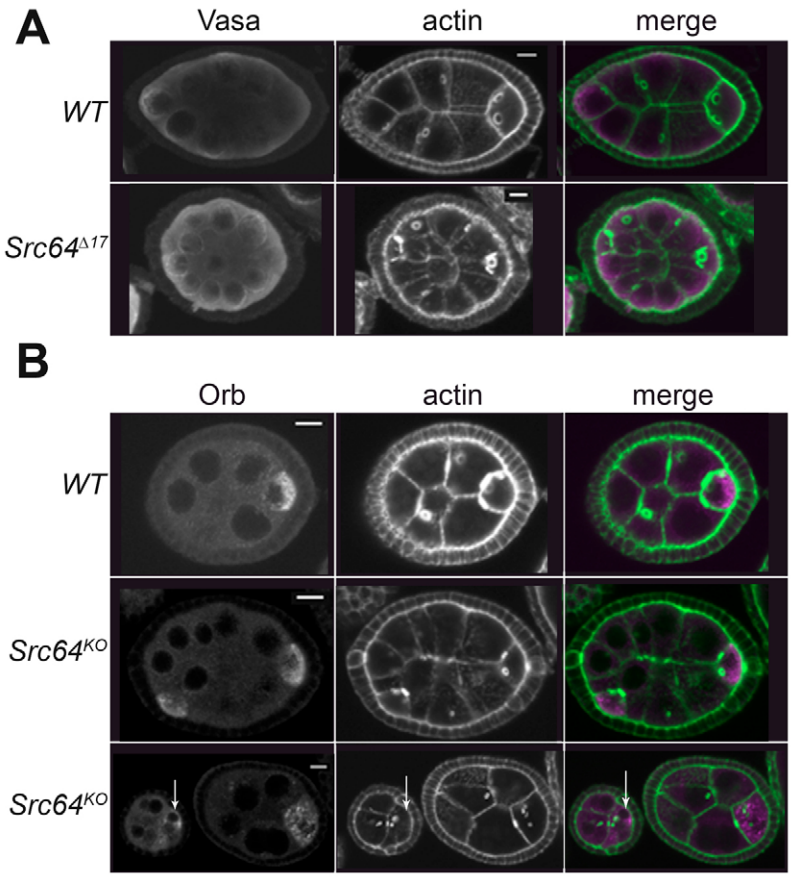

Fig. 6. Germ cell differentiation and oocyte specification are normal in *Src64* mutants. (**A**) Wild-type or $Src64^{\Delta 17}$ egg chambers stained with phalloidin (green) to visualize filamentous actin and anti-Vasa (purple). (**B**) WT or $Src64^{KO}$ egg chambers labeled with phalloidin (green) and anti-Orb (purple). $Src64^{KO}$ oocytes are normally labeled with anti-Orb (middle), but leaking of Orb from the oocyte (arrow) is seen when the cortical membrane breaks down (bottom). Scale bars: 10 μm.

differentiation of specialized subpopulations of follicle cells, including the stalk cells, which are crucial for proper packaging (Ruohola et al., 1991; Bilder et al., 2000; Grammont and Irvine, 2001; Lopez-Schier and St. Johnston, 2001). In the absence of stalk cells, adjacent egg chambers can collapse into one another, forming fused egg chambers (Torres et al., 2003). Finally, the maintenance of follicle cell morphology, and particularly the apical-basal organization of the nascent epithelium, is essential for the cell migration events that control packaging (Goode et al., 1996; Goode and Perrimon, 1997; Abdelilah-Seyfried et al., 2003).

In order to assess follicle cell proliferation in *Src64* mutants, we examined *Src64* egg chambers for gaps in the somatic epithelium that would indicate diminished proliferation rates. However, gaps in the follicle cell layer were not observed in $Src64^{LOF}$ or $Src64^{GOF}$ mutants. Additionally, follicle cells in *Src64* mutants were not aberrantly maintained in an undifferentiated state, as Fas3, a marker for undifferentiated follicle cells in early stages of oogenesis, was downregulated at the appropriate stage (Ruohola et al., 1991; Deng et al., 2001; Lopez-Schier and St. Johnston, 2001) (Fig. 7A).

Furthermore, polar and stalk cell differentiation occurred normally in *Src64* mutants. Stalk formation depends on a Delta-Notch signaling relay system that specifies anterior polar cells, which then induce stalk cell formation (Torres et al., 2003). Egg chambers lacking *Notch* in the follicle cells or *Delta* in the germline fail to produce polar and stalk cells and exhibit dramatic packaging defects (Ruohola et al., 1991; Xu et al., 1992; Lopez-Schier and St. Johnston, 2001; Torres et al., 2003). To assess whether the germline-specific requirement for *Src64* in packaging is mediated through control of Delta/Notch signaling and/or stalk cell specification, we examined *Src64* mutants for downstream markers of Delta/Notch signaling. These markers include Cut, which is expressed in polar and stalk cells (de Celis et al., 1996; de Celis and Bray, 1997; Sun and Deng, 2005), Fas3, which localizes to polar cells by stage 3 (Ruohola et al., 1991), or the β-galactosidase-expressing enhancer trap line B1-93F, which marks stalk cells (Ruohola et al., 1991). Expression of each marker in wild-type, $Src64^{KO}$ or [$Src64^{Y547F}$] ovarioles was indistinguishable (Fig. 7A-C). Additionally, *Src64* egg chambers were always separated by stalks, even when egg chambers were mispackaged (Fig. 7), indicating that specification of the polar and stalk cells was normal in $Src64^{LOF}$ and $Src64^{GOF}$ mutants.

We next examined whether the polarity of the follicle cells was altered in *Src64* germaria. Localization of proteins that mark apical (Baz), basolateral (Arm) and basal (βPS integrin) domains (Tepass et al., 2001) were identical in wild-type and $Src64^{KO}$ mutants (Fig. 7D,E and data not shown). Similar results were seen in [$Src64^{Y547F}$]-expressing germaria (Fig. 7D,E), demonstrating that the polarity of the follicle cells is normal when Src64 levels are altered.

Src64 mutants exhibit defects in the germarium during packaging

To further define the timing of the initial *Src64* defect, we analyzed *Src64* germaria for potential packaging defects. The encapsulation of cysts in wild-type germaria can be visualized by labeling follicle cells and their projections with anti-Fas3 antibodies and germline cysts with anti-Vasa antibodies. In wild-type germaria, 16 germ cells are always surrounded by a single layer of epithelial follicle cells, and follicle cell projections extend to the anterior of flattened germline cysts (Fig. 7F). However, in $Src64^{\Delta 17}$, $Src64^{KO}$, or [$Src64^{Y547F}$] germaria, cysts with aberrant cell numbers were frequently observed (Fig. 7G-I), and, in rare cases, individual germ

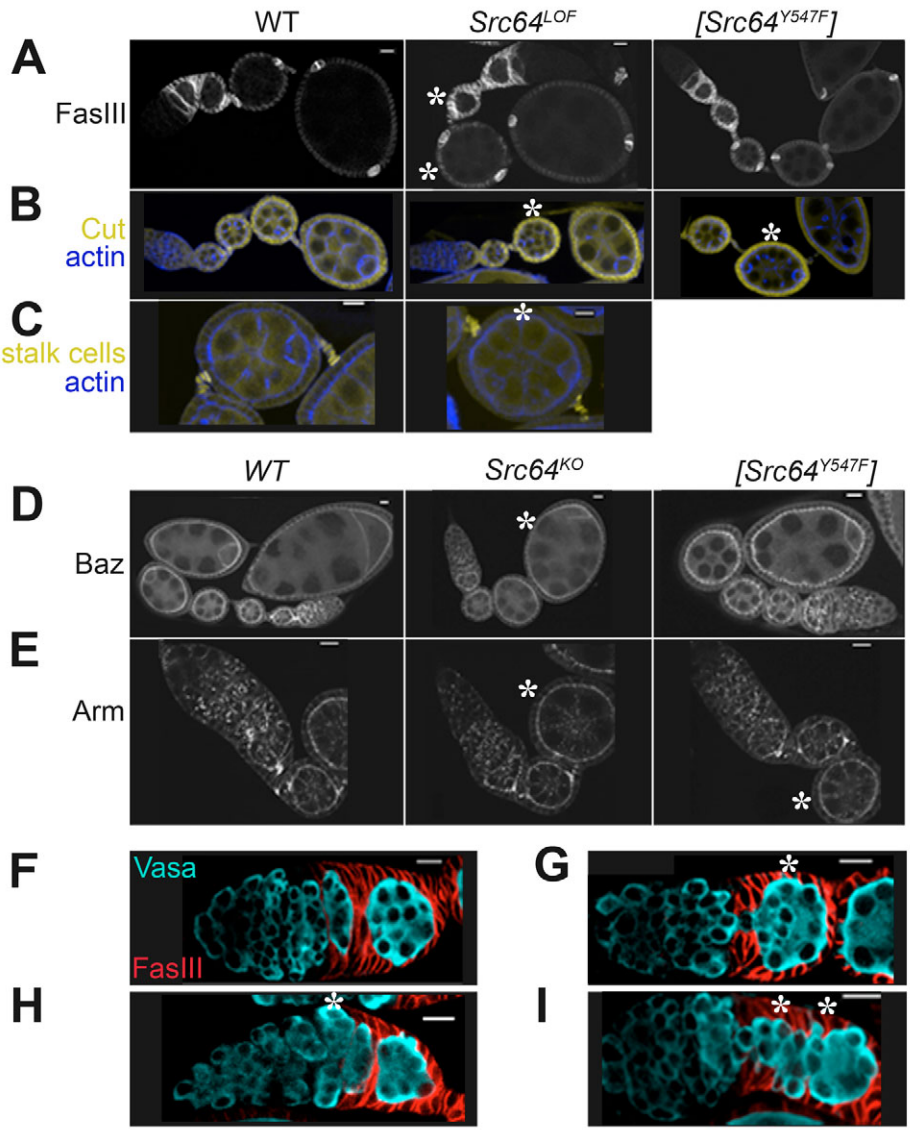

Fig. 7. Follicle cells are normal in *Src64* mutants. Mispackaged egg chambers are indicated by asterisks in all panels. Wild type (WT), $Src64^{LOF}$ ($Src64^{KO}$ or $Src64^{\Delta 17}$), or [$Src64^{Y547F}$] egg chambers labeled with (**A**) anti-FasIII, (**B**) anti-Cut (yellow) and phalloidin (blue), (**C**) WT (w; P[w+BL-93FlacZ]/+) or $Src64^{\Delta 17}$ (w; $Src64^{\Delta 17}$, P[w+BL-93FlacZ]/$Src64^{\Delta 17}$) egg chambers labeled with anti-β-gal (yellow) to visualize stalk cells and phalloidin (blue). (**D**) anti-Baz (apical marker). (**E**) anti-Arm (lateral marker). (**F-I**) Germaria labeled with anti-Vasa (teal) and anti-FasIII (red). (F) *WT*, (G) $Src64^{\Delta 17}$, (H) $Src64^{KO}$, (I) [$Src64^{Y547F}$].

cells were separated from the rest of the cyst (Fig. 7G). The follicle cells in $Src64^{LOF}$ germaria extended projections and migrated to fully surround germline cysts, supporting the idea that *Src64* mutation does not affect follicle cell function. Germline cysts in $Src64^{KO}$ germaria often failed to flatten in region 2B, resulting in the simultaneous packaging of side-by-side cysts (Fig. 7H), a defect that probably contributes to faulty packaging. The penetrance of $Src64^{\Delta 17}$ packaging defects within germaria paralleled that observed in vitellaria (Tables 2, 5), suggesting that later defects are a consequence of aberrations in initial encapsulation. $Src64^{KO}$ and [$Src64^{Y547F}$] mutants exhibited a higher penetrance of packaging defects in germaria versus vitellaria (Tables 2, 5), a discrepancy that is probably due to the difficulty of scoring mispackaging in older egg chambers with collapsed cortical membranes. Together, these data indicate that *Src64* mutant packaging defects occur before the specification of polar and stalk cells and are distinct from these events. Instead, *Src64* mutant packaging defects occur while follicle cells are invading the germarium to surround germline cysts, suggesting that defective communication or adhesion between cells may occur in $Src64^{LOF}$ mutants.

Src64 is activated on ring canals and at follicle cell-germ cell contacts

To better understand the role of Src64 during packaging, we examined Src64 activation patterns during this process. Upon activation, many SFKs are autophosphorylated on a tyrosine in the kinase domain activation loop (Brown and Cooper, 1996). Antibodies that specifically recognize this phosphorylated tyrosine (Y416 in chicken c-Src) have been used as markers for SFK activation (Miermont et al., 2000). We generated and purified antibodies against the analogous phosphorylated tyrosine, pY434 in Src64, and tested their specificity. The antibody specifically recognized pY434, as S2 tissue culture cells expressing [$Src64^{WT}$] or [$Src64^{Y547F}$] transgenes stained with anti-pY434 antibodies, whereas cells expressing [$Src64^{Y434F/Y547F}$], a version of Src64 that is open but lacking the autophosphorylation site, did not (data not shown).

Ring canals in wild-type egg chambers stained robustly with anti-pY434 throughout oogenesis (Fig. 8A), consistent with known roles for Src64 at ring canals (Dodson et al., 1998). Anti-pY434 ring canal staining was eliminated by competition with the pY434 peptide antigen, whereas incubation with the unphosphorylated peptide

Table 5. Quantitation of *Src64* packaging defects in germaria

Genotype	Percentage of germaria with packaging defects (%)	Standard deviation (%)	Number germaria scored (*n*)	Statistical relationships*
w^{1118}	6.5	2.5	250	†
$w;Src64^{KO}$	50.6	9.8	342	‡
$w;Src64^{\Delta 17}$	59.2	4.1	279	‡
$w;[UASpSrc64^{Y547F}]/nosGal4VP16$	41.6	7.0	224	‡

*Differences between † and ‡ were statistically significant, as determined by Student's *t*-test ($P<0.001$).

(Y434) or an unrelated pY peptide had minimal effects (data not shown). Finally, no anti-pY434 signal was observed in either $Src64^{\Delta 17}$ or $Src64^{KO}$ mutants (Fig. 8B, Fig. 9A and data not shown), demonstrating that Src64 expression is required for the pattern observed. Germline expression of [$Src64^{WT}$] rescued anti-pY434 ring canal staining in $Src64^{LOF}$ mutants, but egg chambers expressing a catalytically inactive version of Src64 ([$Src64^{KD}$]) exhibited no staining (Fig. 8B). These experiments suggest that anti-pY434 antibodies can be used to mark sites of Src64 activation in vivo.

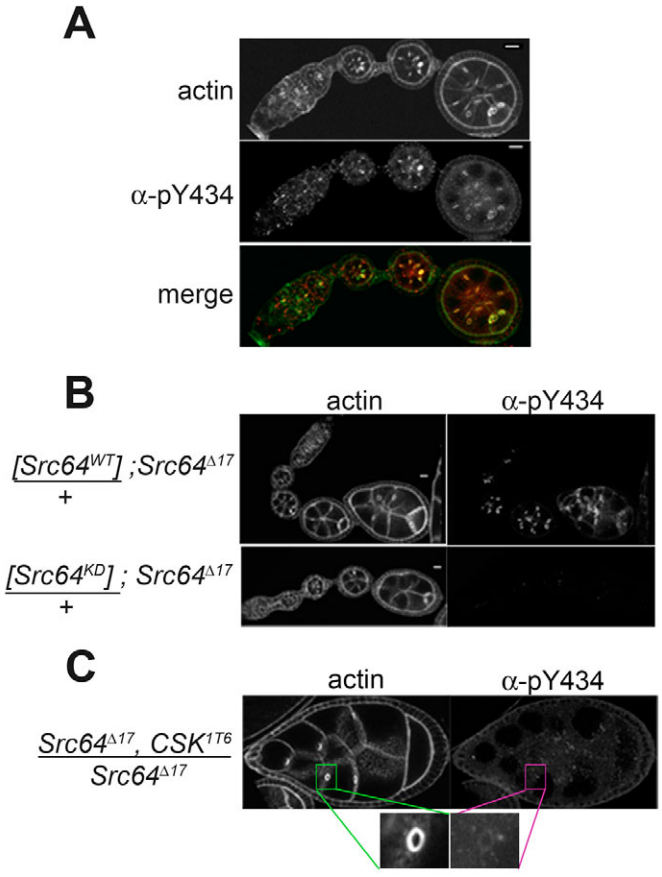

Fig. 8. Src64 is activated on ring canals. (**A**) Wild-type ovariole stained with phalloidin to visualize filamentous actin (green) and anti-pY434 activation-specific Src64 (red). (**B**) Rescue of Src64 activation (right) by expressing wild-type ([$Src64^{WT}$]/+;$Src64^{\Delta 17}$), but not kinase dead ([$Src64^{KD}$]/+;$Src64^{\Delta 17}$) *Src64* in $Src64^{\Delta 17}$ mutants. Filamentous actin is labeled with phalloidin (left). (**C**) Reducing *Csk* in *Src64* hypomorphs can suppress the loss of Src64 activation at the ring canal. $Src64^{\Delta 17}, Csk^{1T6}/Src64^{\Delta 17}$ egg chamber stained with phalloidin and anti-pY434. Boxes (green or pink) surround a ring canal that is weakly stained with anti-pY434. Below is a larger magnification view of the same ring canal.

We next assessed whether reducing Csk function in wild-type or $Src64^{LOF}$ backgrounds affected anti-pY434 staining patterns or levels. We were unable to detect increased anti-pY434 staining on ring canals in *Csk* mutant ovaries with wild-type *Src64* levels. By contrast, removing one copy of *Csk* in $Src64^{\Delta 17}$ mutants ($Src64^{\Delta 17}, Csk^{1T6}/Src64^{\Delta 17}$,+) enabled us to detect weak anti-pY434 staining on some ring canals (Fig. 8C). No anti-pY434 ring canal staining was observed when one copy of Csk was removed in $Src64^{KO}$ mutants (data not shown). These results are consistent with our observation that reducing *Csk* levels suppressed *Src64* ring canal growth defects when Src64 protein levels were limiting (Table 2) and support a model in which Csk directly regulates Src64.

To address whether Src64 is activated during packaging, we examined the staining patterns of anti-pY434 antibodies in germaria. In addition to clear ring canal staining, a punctate rim of staining was observed at junctions between germline cysts and follicle cells during encapsulation (Fig. 9A) and in the developing stalk region. Similar staining patterns were seen when the follicle cells lacked *Src64* (Fig. 9B), but no staining of either ring canals or cell-cell contacts was observed in $Src64^{LOF}$ germaria (Fig. 9A) or in $Src64^{LOF}$ germline clones (Fig. 9B). These results support the idea that Src64 functions in the germ cells and raise questions about potential roles for germline signals during stalk formation. Staining of the follicle cell-germ cell contact region persisted until stage 2, and then became dramatically diminished in older egg chambers. This pattern of activation, combined with the role of Src64 in regulating packaging, raises the possibility that Src64 participates in dynamic rearrangements of cell contacts during packaging, and that its activity is no longer required once egg chamber assembly is completed.

DISCUSSION

Many studies have focused on roles for SFKs in regulation of proliferation, differentiation and dynamic changes in cellular morphology. In this report, we show that Src64 is dispensable for proliferation and differentiation of both germ cells and follicle cells in the *Drosophila* ovary. Instead, Src64 is required for morphological changes at the ring canal and contributes to the packaging of germline cysts by follicle cells during egg chamber formation. Our results demonstrate that Csk regulates Src64 function during packaging, but is dispensable during ring canal growth control. Thus, regulation of Src64 activity levels during these two morphological events is distinct.

Actin polymerization is a crucial component of ring canal growth regulation (Hudson and Cooley, 2002), and mutation of genes that control actin dynamics causes dramatic ring canal defects (Hudson and Cooley, 2002; Somogyi and Rorth, 2004). $Src64^{\Delta 17}$ ring canals are smaller than wild type (Dodson et al., 1998) and exhibit diminished actin polymerization (Kelso et al., 2002). Recent work has shown that Src64-mediated phosphorylation of the actin-bundling protein Kelch is crucial for regulating actin polymerization during ring canal growth. Whereas the $Src64^{\Delta 17}$ ring canal defects

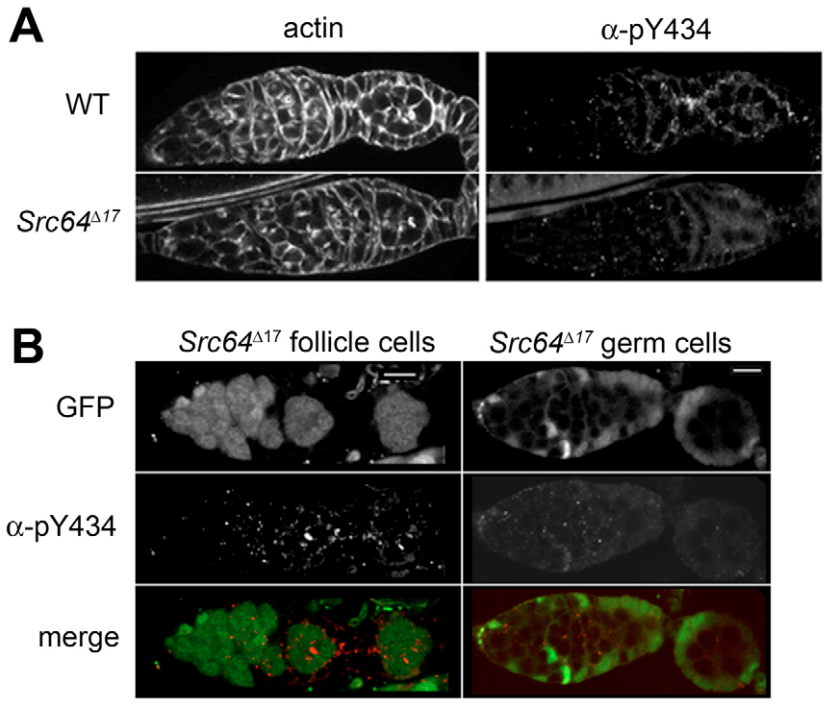

Fig. 9. Anti-pY434 labels contact points between germ cells and follicle cells in germaria. (**A**) Wild-type (WT) or *Src64*$^{\Delta 17}$ germaria stained with phalloidin to label filamentous actin or anti-pY434. (**B**) *Src64*$^{\Delta 17}$ mutant follicle cells (left) or germ cells (right) are marked by the absence of GFP (green). Anti-pY434 stains ring canals and follicle cell-germ cell contacts only when the germ cells express *Src64*. Scale bars: 10 μm.

are strikingly similar to those observed in germ cells expressing only [Kelch ^{YA}], which cannot be tyrosyl phosphorylated by Src64 (Kelso et al., 2002), we found that *Src64*^{KO} ring canal growth defects are more severe than those in *Src64*$^{\Delta 17}$ (Fig. 2, Table 1) or, by inference, [*Kelch*^{YA}] mutants. This result suggests that *Src64* may control additional signals during this process. Cortactin or members of the WASP/SCAR protein family promote actin polymerization through Arp2/3 complex activation (Weaver et al., 2003) and are required for ring canal growth regulation (Somogyi and Rorth, 2004; Zallen et al., 2002). Both types of protein are known vertebrate SFK substrates (Brunton et al., 2004), suggesting the possibility that several Src64-dependent routes may drive the actin polymerization required for ring canal growth.

Src64 is active on ring canals throughout oogenesis (Figs 8, 9), consistent with known requirements for Src64 kinase activity during ring canal growth (Lu et al., 2004). The ring canal-specific pattern of activated Src64 staining contrasts with the localization of Src64 protein to all germ cell membranes (Dodson et al., 1998), (Figs 1, 8, 9), suggesting that Src64 activators are present specifically at ring canals. SFKs can be activated either through SH3-SH2 domain binding to ligand or PTP-mediated dephosphorylation of the C-terminal regulatory tyrosine (Brown and Cooper, 1996). Csk opposes PTP action by phosphorylating the SFK C-terminal tyrosine, thus promoting the inactive state. If the primary mechanism that determines Src64 activation at the ring canal is PTP-mediated dephosphorylation, we would expect that loss of Csk should have dramatic effects on ring canal growth. However, we found no significant effects on ring canal growth in germ cells lacking *Csk* (Table 2) or that express a version of *Src64* that cannot be regulated by Csk ([Src64^{Y547F}], Table 1). Our results suggest that a minimum threshold of Src64 activity is required for regulation of ring canal growth and, once this threshold is reached, the Src64-mediated response is saturated. Consistent with this idea, reduction of Csk function can suppress *Src64* mutant defects and partially restore Src64 activation under limiting Src64 conditions (Table 2, Fig. 8C). Taken together, these results suggest that Src64 is predominantly regulated by SH3-SH2 domain engagement at the ring canal and that Csk plays a minor role in this process.

In addition to *Src64* ring canal defects, deviation from wild-type Src64 activity levels leads to the formation of egg chambers containing aberrant germ cell numbers surrounded by a normal follicular epithelium (Fig. 4) (Djagaeva et al., 2005). Egg chambers containing incorrect germ cell numbers can arise due to germ cell or follicle cell proliferation defects, failure to properly differentiate the stalk cells that separate adjacent egg chambers, or as a result of defective packaging of germline cysts by follicle cells within the germarium (de Cuevas et al., 1997; Horne-Badovinac and Bilder, 2005). In this work, we show that both *Src64*^{LOF} and *Src64*^{GOF} mutants exhibit normal proliferation patterns in both follicle cells and germ cells, and that follicle cell polarity and differentiation are unaffected by *Src64* mutation. Instead, defects in the initial separation of germline cysts by invading follicle cells are responsible for *Src64* mutant packaging defects.

Two previously identified genes, *egghead* (*egh*) and *brainiac* (*brn*) are required in the germline to regulate the migration of follicle cell precursors during packaging (Goode et al., 1996). When germ cells lack *egh* or *brn*, follicle cell precursors frequently fail to extend projections, leading to the packaging of multiple germline cysts into one compound egg chamber. Mutations in *egh* or *brn* also affect follicle cell polarity and later migration events. Similarly, genes such as *Delta*, *toucan* or *BicD* are involved in germline-derived signals that affect follicle cell differentiation or morphogenesis (Grammont et al., 1997; Lopez-Schier and St. Johnston, 2001; Oh and Steward, 2001). These results suggest that instructive cues generated by the germ cells direct follicle cell morphogenesis during packaging.

Although *Src64* is required in the germ cells, *Src64* mutant phenotypes are inconsistent with a similar role for Src64 in regulating follicle cell morphogenesis. No defects in follicle cell proliferation, process extension, migration, differentiation or polarity are observed in *Src64* mutants. Importantly, Src64 is activated at contact points between germ cells and follicle cells while

packaging occurs (Fig. 9). This finding implies that contact between follicle cells and germ cells leads to changes in the germ cell surface over which follicle cells migrate, indicating that germ cells actively respond to follicle cell-derived signals. Roles for SFKs in dynamic regulation of endothelial cell surfaces that act as substrata for attachment and migration of leukocytes or metastatic tumor cells have been previously proposed (Eliceiri et al., 1999; Weis et al., 2004). In endothelial cells lacking SFK activity, leukocyte attachment and migration is defective, and metastatic colon cancer cells fail to penetrate the endothelial barrier. These results demonstrate crucial roles for SFKs in establishing an appropriate substratum for cell migration.

We propose that Src64 functions in an analogous manner during packaging. In this model, Src64 is activated by contact between follicle cell projections and germ cells. The precise Src64 activity levels are determined by the balance between contact-dependent activators and Csk. Src64-dependent activation of downstream pathways may then establish the germ cell surface as an appropriate substratum for follicle cell attachment and migration. Defects in adhesion or the underlying cytoskeleton resulting from inappropriate Src64 activation levels would lead to defective adhesion by invading follicle cells, resulting in packaging defects.

E-cadherin and Arm/β-catenin are important regulators of adhesion between germ cells within an individual cyst as well as adhesion between germ cell and follicle cell surfaces. Germline mutation of *arm* or *shotgun* (*shg*), which encodes E-cadherin, leads to ring canal attachment defects, failure of germline cysts to flatten across the germarium, packaging defects and oocyte mislocalization (Peifer et al., 1993; Oda et al., 1994; Godt and Tepass, 1998; Gonzalez-Reyes and St. Johnston, 1998). These phenotypes overlap with *Src64* mutant defects, suggesting that Src64 might function within germ cells to regulate E-cadherin complexes. Vertebrate SFKs can dynamically alter the adhesive strength of E-cadherin-mediated complexes through catenin phosphorylation (Brunton et al., 2004; Reynolds and Roczniak-Ferguson, 2004; Lilien and Balsamo, 2005), supporting the idea that Src64 may function similarly during oogenesis. Although direct regulation of E-cadherin-mediated adhesion by Src64 is an attractive model, we did not observe changes in the levels of E-cadherin or Arm at germ cell or follicle cell membranes in *Src64* mutants (Fig. 7E and data not shown), *shg* is dispensable for Src64 activation (A.M.O. and M.A.S., unpublished), and the most prominent phenotype observed in *shg* or *arm* mutants is oocyte mislocalization (Peifer et al., 1993; Godt and Tepass, 1998; Gonzalez-Reyes and St. Johnston, 1998), a phenotype that occurs in less than 1% of *Src64* mutant egg chambers (Djagaeva et al., 2005) (data not shown). It is possible that Src64 selectively regulates E-cadherin complexes that mediate ring canal attachment and the germ cell-follicle cell interactions that occur during packaging without affecting oocyte localization. Alternatively, Src64 may target a different adhesion complex, the disruption of which indirectly affects E-cadherin-dependent events. Further analysis of the relationships between Src64 and E-cadherin complex members is required to distinguish between these possibilities.

The incomplete penetrance of packaging defects in *Src64* mutants suggests that follicle cells can package germline cysts properly even when an ideal substratum is lacking, that Src64 plays a modifying role in this process, or that additional unidentified mechanisms function redundantly with Src64-controlled events. Future identification of upstream activators and downstream consequences of Src64 activation will contribute significantly to the understanding of its role in regulating the germ cell surface during packaging.

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