Requirements of genetic interactions between *Src42A*, *armadillo* and *shotgun*, a gene encoding E-cadherin, for normal development in *Drosophila*

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Summary

***Src42A* is one of the two *Src* homologs in *Drosophila*. *Src42A* protein accumulates at sites of cell-cell or cell-matrix adhesion. Anti-Engrailed antibody staining of *Src42A* protein-null mutant embryos indicated that *Src42A* is essential for proper cell-cell matching during dorsal closure. *Src42A*, which is functionally redundant to *Src64*, was found to interact genetically with *shotgun*, a gene encoding E-cadherin, and *armadillo*, a *Drosophila* β-catenin. Immunoprecipitation and a pull-down assay indicated that Src42A forms a ternary complex with E-cadherin and Armadillo, and that Src42A binds to Armadillo repeats via a 14 amino acid region, which contains the major autophosphorylation site. The leading edge of *Src* mutant embryos exhibiting the dorsal open phenotype was frequently kinked and associated with significant reduction in E-cadherin, Armadillo and F-actin accumulation, suggesting that not only Src signaling but also *Src*-dependent adherens-junction stabilization would appear likely to be essential for normal dorsal closure. Src42A and Src64 were required for Armadillo tyrosine residue phosphorylation but Src activity may not be directly involved in Armadillo tyrosine residue phosphorylation at the adherens junction.**

Key words: Dorsal closure, Adherens junction, *Src42A*, *arm*, *shotgun*, E-cadherin

Introduction

The vertebrate Src family of non-receptor tyrosine kinases comprises nine members, three of which, Src, Yes and Fyn, are widely expressed in a variety of cells (reviewed by Thomas and Brugge, 1997). These Src kinases are considered to have crucial roles in modulation of the actin cytoskeleton, a determinant of cell-shape change and cell migration. Transformation of fibroblasts with activated Src kinases gave rise not only to actin-cytoskeleton disruption (Boschek et al., 1981) but also increased tyrosine phosphorylation of many cytoskeleton-associated proteins involved in cell-substratum and cell-cell interactions (Brown and Cooper, 1996). The importance of Src kinases as regulators of cell migration and cell-shape change is also underscored by studies using fibroblasts derived from mice deficient in Src, Yes and Fyn (Klinghoffer et al., 1999).

The major autophosphorylation site in focal adhesion kinase (FAK) serves as a binding site for Src homology 2 (SH2)-containing proteins (Chen et al., 1996; Schaller et al., 1994; Schlaepfer et al., 1999). The FAK-Src complex mediates the phosphorylation of paxillin and p130-Crk-associated substrate, both of which are major scaffolding proteins capable of recruiting other molecules for integrin-based cell-substratum adhesions and which regulate cytoskeleton organization (Bellis et al., 1995; Cary et al., 1998; Honda et al., 1998; Honda et al., 1999; Schaller et al., 1995; Turner, 2000). The absence of FAK gave rise to increase in the number and extent of cell-substratum adhesions (Ilic et al., 1995). Recently, quantitative assay of the rate of incorporation of proteins into cell-substratum adhesion and departure of these proteins from this adhesion was conducted (Webb et al., 2004). Src and FAK were shown to be crucial for adhesion turnover at the cell front. Thus, the rates of formation, disassembly and/or maturation of cell-substratum-adhesion appear controlled by FAK-Src activity.

Homophilic cadherin interaction is essential for cell-cell adhesion in vertebrates (Hinck et al., 1994). The loss of E-cadherin (E-cad) expression has been shown related to invasive and metastatic cancers (Denk et al., 1997; Van Aken et al., 2001). β-Catenin binds to α-catenin and the cytoplasmic domain of E-cad and is essential for linking E-cad to the actin cytoskeleton (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). Tyrosine-phosphorylation of β-catenin or other adherens-junction-associated proteins is one means by which cadherin-mediated cell-cell adhesions may be altered (Lilien et al., 2002; Takeda et al., 1995). Enhanced tyrosine-phosphorylation of β-catenin causes weakening of cadherin-actin interaction with consequent loss of cell adhesiveness. Src may be one of the tyrosine kinases responsible for this tyrosine-phosphorylation, because, in cells transformed with

Src, loss of epithelial cell differentiation, gain in invasiveness and cadherin-mediated adhesion detachment are all correlated with tyrosine-phosphorylation of the E-cad/β-catenin complex (Behrens et al., 1993; Hamaguchi et al., 1993; Lilien et al., 2002).

Nonetheless, precise determination of the functional roles of individual Src family kinases in vertebrates may be attended with considerable difficulty in that compensatory interactions may occur among nine vertebrate Src kinase members. By contrast, *Drosophila* possesses only two Src kinases, Src64 and Src42A (Simon et al., 1985; Takahashi et al., 1996) and, accordingly, may provide a better and simpler system for clarifying Src functions in development.

Mutations in *Src64* lead to reduction in female fertility, which is associated with nurse cell fusion and ring canal defects (Dodson et al., 1998). *Src64*-mutant ring canals fail to undergo extensive tyrosine phosphorylation which normally occurs. *Tec29* dominantly enhances the *Src64* ring canal phenotype and loss of *Tec29* results in a phenotype strikingly similar to that noted following loss of *Src64* function (Guarnieri et al., 1998). Tec29 kinase is localized in the ring canal, and this subcellular localization requires *Src64* function, indicating that Tec29 is a downstream target of Src64.

Src42A is the closest relative of vertebrate *Src* in *Drosophila*. By localized expression of gain-of-function and dominant-negative forms of *Src42A*, it was demonstrated that Src42A may be involved in the regulation of cytoskeleton organization and cell-cell contacts in developing ommatidia and that both dominant-negative and gain-of-function mutations of *Src42A* cause formation of supernumerary R7-type neurons, which is suppressible by one-dose reduction of various components involved in the Ras/MAPK pathway (Takahashi et al., 1996). Lu and Li (Lu and Li, 1999) isolated a *Src42A* mutant as an extragenic suppressor of *Raf* and with this and other mild *Src42A* mutants found that Src42A may serve as negative regulator of receptor tyrosine kinases in a Ras1-independent manner. Their genetic data for Src functions in ommatidium formation appeared somewhat at variance with those of Takahashi et al. (Takahashi et al., 1996) using gain-of-function and dominant-negative types of *Src42A* transgenes.

As with *Src64*, *Src42A* may function in a synergistic manner with *Tec29*. A *Tec29* mutation was noted to enhance the lethality of *Src42A* mutants dominantly (Tateno et al., 2000). Although these authors found no dorsal open phenotype in their *Src42A* or *Tec29* mutants, the double mutant embryos exhibited the dorsal open phenotype. *Src42A* has been shown to be functionally redundant to *Src64* at least in the dorsal closure (Tateno et al., 2000). Both dorsal closure of the embryonic epidermis and thorax closure of the pupal epidermis require the Jun amino-terminal kinase (JNK) homolog Basket (Bsk) (Zeitlinger and Bohmann, 1999; Tateno et al., 2000). The severity of the epidermal closure defect in *Src42A* mutants was found to depend on the degree of *Bsk* activity, and this extent to depend on that of *Src42A* (Tateno et al., 2000), thus indicating that JNK-pathway activation is required downstream of Src42A.

This paper first presents dynamic changes in cellular and subcellular localization of Src42A and then describes phenotypes of a *Src42A* protein-null and *Scr42A Src64* mutants. Genetic and biochemical analyses indicate that E-cad and Armadillo (Arm) form a complex with Src in the membrane and the resultant putative adherens junction complex is required for proper regulation of F-actin accumulation and actin cytoskeleton dynamics in leading edge cells during dorsal closure.

Materials and methods

Plasmids and *Drosophila* strains

Plasmids used were: metallothionein-Gal4 (Brand and Perrimon, 1993), pMAL-c2X (New England BioLabs), pGEX6P-1 (Amersham Biosciences), pGST-Arm, pUAS-Arm, pUAS-Src42A[KR], pUAS-Src42A[WT], pUAS-Src42A[YF], pMBP-Src42A[SH3SH2], pMBP-Src42A[Kinase[KR]], pMBP-Src42A[391/404] and pMBP-Src42A[(Kinase)-(391/404)]. pUAS-Arm was constructed by introducing the Arm-coding region into the *Xho*I-*Not*I site of pUAST (Brand and Perrimon, 1993), while pUAS-Src42A[KR], pUAS-Src42A[WT] and pUAS-Src42A[YF] were generated by inserting dominant-negative, wild-type or constitutively active forms of the *Src42A* gene (Takahashi et al., 1996) into the *Not*I site of pUASV (Sato et al., 1999). pMBP derivatives were constructed by introducing *Src42A* fragments (see Fig. 7D) into the *Eco*RI-*Sal*I site of pMAL-c2X. pGST-Arm is a pGEX6P-1 derivative encoding Arm repeats (amino acids 140 to 671). Fly strains used were: Canton S (wild-type), shg^{R64a}, shg^{g317}, shg^{R69}, shg^{R6} (Niewiadomska et al., 1999; Tepass et al., 1996), $arm^{H8.6}$, arm^{YD35} (Peifer and Wieschaus, 1990), $Src64^{P1}$ (Tateno et al., 2000), *pannier* (*pnr*)-GAL4 (Calleja et al., 1996) and $Src42A^{6-1}$ (M.T., unpublished). UAS-*Src42A[KR]*, UAS-*Src42A[WT]* and UAS-*Src42A[YF]* flies were generated essentially according to Spradling and Rubin (Spradling and Rubin, 1982). Other strains are described in FlyBase. *Src* double mutant embryos were identified by expression of Src42A and *lacZ* markers on balancer chromosomes.

Screening of dominant enhancers for *sev-Src42A[KR]*

Enhancers were searched for using P[*sev-Src42A[KR]*] (Takahashi et al., 1996) inserted into CyO or TM3 balancers. E(7A-1), which is incapable of complementing shg^{R64a}, was isolated from 20,000 EMS-mutagenized flies.

Antibodies

Histochemical reagents and primary antibodies used were: TUNEL reagents (Roche Diagnostics), rhodamine-phalloidin, SYTOX (Molecular Probes), mouse anti-tracheal lumen (2A12) (Manning and Krasnow, 1993), mouse anti-Fas3 (Patel et al., 1987), mouse anti-Fas2 (Hummel et al., 2000), mouse anti-Arm (Riggleman et al., 1990), rat anti-E-cad (Oda et al., 1994), rat anti-Src64 (Dodson et al., 1998), biotinylated mouse anti-phosphotyrosine (pTyr) (Glenney et al., 1988), mouse anti-α-Tubulin (ICN), mouse anti-*lacZ* (Promega), rabbit anti-GST (glutathione S-transferase; Sigma), rabbit anti-MBP (maltose binding protein; New England BioLabs), mouse anti-Elav (Robinow and White, 1991), mouse anti-Engrailed (Patel et al., 1989) and rabbit anti-Clawless (Cll) (Kojima et al., 2005) antibodies. Src42A antiserum (rabbit) was raised against GST-Src42A (amino acids 1-252) fusion protein [see other details in Suzuki and Saigo (Suzuki and Saigo, 2000) and Hayashi et al. (Hayashi et al., 1998)].

Cell culture, RNA interference (RNAi), immunoprecipitation and pull-down assay

Transfection and RNAi of *Drosophila* S2 cells was carried out as described previously (Ui-Tei et al., 2000). Membrane and cytosolic fractions for immunoprecipitation were prepared from stage 13-15 embryos according to Peifer (Peifer, 1993). The pellet (membrane fraction) was resuspended in buffer with the same volume of the cytosolic fraction. Immunoprecipitates were size-fractionated and immunoblotted. Pull-down assay was carried out as follows. MBP-tagged proteins, bacterially expressed, were bound to amylose resin.

After PBT washing, resins were incubated with bacterially expressed GST-tagged proteins. The MBP/GST fusion protein complex was eluted with maltose (10 mM) and analyzed with western blotting with anti-GST or anti-MBP antibodies.

Results

Dynamic changes in Src42A distribution

For assessment of Src42A distribution, ovaries and embryos were stained for Src42A and E-cad. As expected from RNA staining (Takahashi et al., 1996), Src42A signals appeared over the entire plasma membrane of all cells, but strong Src42A signals could often be found at sites of either cell-cell or cell-matrix adhesion.

At the start of oogenesis, the cystoblast undergoes four rounds of mitotic divisions with incomplete cytokinesis to generate 16 cystocytes interconnected via ring canals (Cooley and Robinson, 1996). Follicle cells subsequently separate off individual cysts to form egg chambers.

Transient but very strong Src42A signals, not associated with strong E-cad signals, were found in cystocytes in germarium region 2a/b (Fig. 1A). E-cad signals became evident at slightly later stages. In stage 1-7 egg chambers, relatively strong Src42A signals were apparent along the nurse/follicle cell boundary (Fig. 1A,B) as noted for E-cad (Niewiadomska et al., 1999); Src42A signals on the basal follicle-cell surface were very weak. In the middle of oogenesis, relatively strong Src42A signals were evident in polar and invading border cells (Fig. 1B,D,E). Middle-stage ring-canals were marked by Src42A enclosed by weak E-cad (Fig. 1C1,C2). By stage 7, cytoplasmic Src42A became evident in oocytes. At stage 8, Src42A unassociated with E-cad started being deposited on the oocyte surface and were conspicuous by stage 10b, at which time strong Src42A and E-cad signals could be seen in centripetal cells (Fig. 1F1,F2).

Embryogenesis starts with cleavage (stages 1-4), in which the nucleus undergoes 13 divisions and the nuclei thus produced become arranged in a single layer beneath the egg surface (Hartenstein, 1993). Membranous Src42A signals were evident (Fig. 1I). During cellularization, not only Src42A but also E-cad signals were apparent on the surface of eggs and the membrane extending inwardly (Fig. 1G1-G3). The leading edges of invading membranes are always marked by Src42A but not E-cad. At stage 6, mesoderm generation started by invagination. In stage 7 dorsal cells lying anterior to the cephalic furrow, Src42A distribution was virtually the same as at stage 5 (Fig. 1G3,H1,J), but in more posterior dorsal cells involved in transient furrow formation or posterior midgut invagination, strong Src42A expression associated with strong E-cad signals could be confirmed only in the apical region (Fig. 1H2,J,K). Src42A unassociated with E-cad expression persisted in invaginated mesodermal cells and were evident on the ectoderm/mesoderm interface at stage 9 (Fig. 1L). Apical tips of mesectodermal cells, situated along the ventral midline, showed strong E-cad and Src42A signals (Fig. 1M1,2).

In late developmental stage embryos, there were strong signals of E-cad and Src42A in some tubular structures (Fig. 1N-R). In all cases, E-cad was present only in apical regions, while Src42A varied in location according to tube type (Fig. 1N,P,R). In hindguts covered with thin visceral mesodermal cells (Fig. 1R, inset), Src42A signals were evident in both apical and basal regions (Fig. 1R), whereas Malpighian tubules protruding from hindguts (Fig. 1R) (Skaer, 1993), salivary glands (Fig. 1P) and stomodeum opening (Fig. 1N), none of which having any mesoderm association, all displayed Src42A signals only in apical regions. Basal strong Src42A signals were eliminated when hindgut cells acquired Malpighian-tubule fate (see arrows in Fig. 1R). Similarly, strong basal Src42A signals, separating the ectoderm from mesoderm at the basal clypeus cortex, had varnished with stomodeum formation (Fig. 1N).

During stage 12, tracheal branches develop from invaginated tracheal pits (Manning and Krasnow, 1993). At stage 13, the dorsal trunk anterior has fused with the dorsal trunk posterior of the anterior neighbor to form a long tubular structure. The arrowheads in Fig. 1S show Src42A to be co-localized with juxtaposition E-cad signals. Strong Src42A signals unassociated with E-cad are present in tendon cells to which the muscle system is attached (see the arrows). Dorsal closure is a major morphogenic process in which two epithelial sheets converge to enclose the embryo. At the leading edge, moderate Src42A signals colocalized with strong E-cad (Fig. 1O).

Strong Src42A signals were evident in CNS (Fig. 1T). Longitudinal connectives and commissures stained strongly with anti-Src42A antibody. E-cad signals could be seen only in midline glial cells, mesectodermal derivatives (see the arrowheads). In CNS, nervous system-specific N-cadherin appeared co-expressed with Src42A (Iwai et al., 1997) (data not shown). Strong Src42A signals were present in the brain (Fig. 1Q) and the axon linking the larval eye (Bolwig's organ) to the optic lobe (Fig. 1U). Strong Src42A signals, occasionally associated with E-cad signals, were seen in the gonad (Fig. 1V) (Jenkins et al., 2003).

Isolation of a protein-null *Src42A* mutant and functional redundancy of *Src42A* and *Src64*

As Src possesses multiple functional domains, the isolation of protein-null mutants may be required to clarify the roles of *Src* in development. Short *Src42A* deletion mutants were thus generated through imprecise P-element excision of $Src42A^{k10108}$ (enhancer trap line) and a protein-null lethal mutant, $Src42A^{26\text{-}1}$, was identified using anti-Src42A antibody (Fig. 2A2,A3). In $Src42A^{26\text{-}1}$, a 1.9 kb region containing the putative TATA box, RNA start, the first exon of *Src42A* and the entire *P-lacZ* sequence were deleted (Fig. 2A1). $Src42A^{26\text{-}1}$ embryos showed mild dorsal closure defects (Fig. 2B1,B2). Close inspection of mutant embryos stained for Engrailed revealed occasional segmental misalignment (Fig. 2B3). Lethality and morphological defects in $Src42A^{26\text{-}1}$ were eliminated by introducing the wild-type *Src42A* transgene driven by *arm*-GAL4 (data not shown).

Using $Src64^{P1}$ and $Src42A^{E1}$, *Src42A* and *Src64* have been shown to be functionally redundant to each other with respect to the dorsal closure (Tateno et al., 2000). Using a newly isolated protein-null *Src42A* mutant, we demonstrate that these two Src genes are functionally redundant not only in dorsal closure but in many other development contexts as well.

The dorsal open phenotype associated with head involution defects was exhibited by 34% of $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ embryos (Fig. 2B4). $Src42A^{26\text{-}1}$;$Src64^{P1}$ embryos showed much severer phenotypes with no apparent germ band retraction (Fig. 2B5). No defects could be found in $Src64^{P1}$ (data not shown). CNS

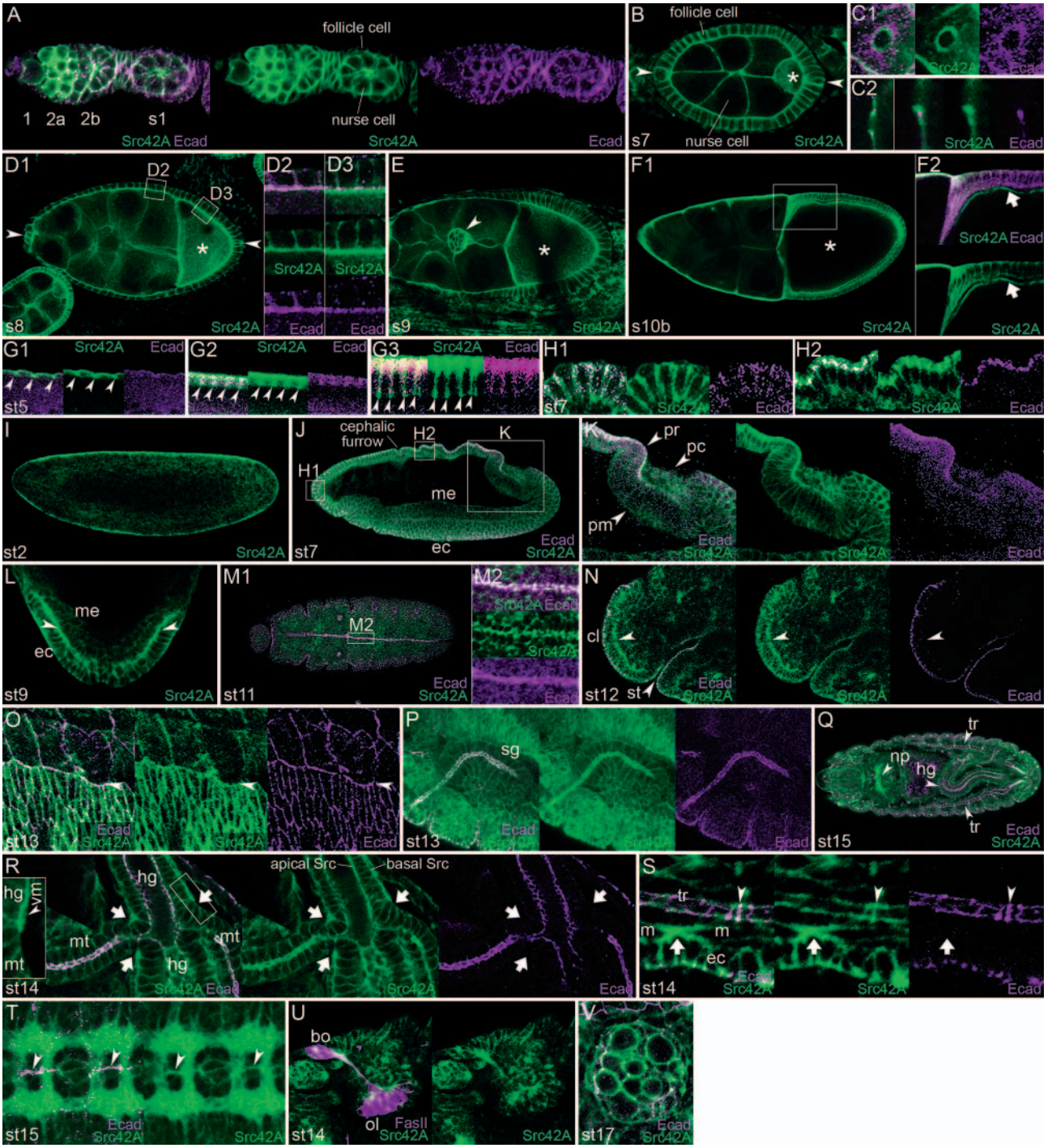

Fig. 1

morphology was extensively affected by the simultaneous elimination of *Src42A* and *Src64* activity. In *Src* double mutant embryos, longitudinal tracts and commissures were frequently broken without significant loss of Elav-positive neuronal cells (Fig. 2C1-C4). In *Src* double mutants, optic lobe/Bolwig's organ (Fig. 2E1-E3) and trachea formation (Fig. 2D1-D4) was significantly disrupted, while no apparent defect was detected in $Src42A^{26\text{-}1}$.

Normal nurse cell formation requires maternal *Src64* activity (Dodson et al., 1998). However, no nurse cell fusion occurred in ovaries doubly heterozygous for $Src42A^{26\text{-}1}$ and $Src64^{P1}$ (Fig. 2F1,F2) and there was hardly any enhancement of *Src64* nurse cell phenotypes such as nurse cell fusion and ring-canal defects with elimination of one copy of *Src42A* (Fig. 2F3,F4). *Src42A* would thus play only minor if any role in ovary development.

Src may thus be considered to exercise central roles in many normal developmental processes of oogenesis and

Fig. 1. Subcellular localization of Src42A. Boxed regions are enlarged in insets or separate panels. (A-F2) Ovaries stained for Src42A (green) and E-cad (magenta). Asterisks indicate oocytes; arrowheads indicate polar or invading border cells. Some cystocytes in region 2a/b exhibited transient but strong Src42A expression (A). In subsequent stages, relatively strong Src42A signals associated with E-cad signals became evident along the follicle/germline-cell boundary (D2). E-cad signals are shown only in enlarged panels. Nurse cell membrane E-cad signals surround ring-canal-Src42A signals (C1,C2). During oogenesis stages 6-9, oocytes incorporated Src42A into the cytoplasm (B,D,E) and began accumulating it on the oocyte surface (D2,D3). (F1,2) Membrane localization of E-cad-free Src42A was clearly seen at stage 10b, when centripetal cells strongly expressed Src42A and E-cad (arrow indicates oocyte membrane). (G1-V) Embryos stained for Src42A and E-cad. Src42A and E-cad signals are colored in green and magenta, respectively (except in U). Stages are shown in the bottom left-hand corner. In stage 2 embryos, membranous Src42A (I) signals scarcely associated with E-cad. (G1-3) Panels showing three phases of membrane extension in stage 5 embryos. Arrowheads indicate extending membrane tips marked with Src42A. (J) At stage 7, apical signals for Src42A and E-cad in furrow-forming or invaginating cells (H2,K) became stronger than in others (H1). At mid-late-stages, E-cad-free Src42A expression occurred between ectodermal and mesodermal cell-layers (see arrows in L). Src42A and E-cad signals colocalized at the apical tips of mesectoderm cells (M1,M2) and the apical surface of ectodermal tubular structures such as stomodeum (N), salivary gland (P), hindgut (Q,R) and Malpighian tubules (R). Malpighian tubules are hindgut protrusions (Skaer, 1993) and, unlike hindgut enclosed with the visceral mesoderm (inset in R), are not associated with visceral mesoderm. Arrows in R show that basal Src42A signals abruptly have disappeared with Malpighian-tubule protrusion. (O) Src42A and E-cad signals at stage-13 leading edge. (S) E-cad/Src42A co-localization at fusion points of dorsal trunk of trachea (see arrowheads). Arrows, Src42A signals in the tendon, rich in integrin (Martin-Bermudo and Brown, 1996). (T,U) E-cad and Src42A expression in CNS. E-cad signals were detected only in midline cells (see arrowheads). Fas2 expression (magenta) (U) shows Src42A expressed in axons, optic lobe and Bolwig's organs. (V) Src42A signals in gonad. hg, hindgut; np, neuropile; pc, pole cells; pm, posterior-midgut primordium; pr, proctodeum primordium; st, stomodeum; tr, trachea; vm, visceral mesoderm; cl, clypeus; ec, ectoderm; me, mesoderm; sg, salivary gland; mt, Malpighian tubule; m, muscle; bo, Bolwig's organ; ol, optic lobe.

embryogenesis. *Src42A* and *Src64* contribution to total *Src* activity would depend on some particular aspect of development.

shotgun and *arm* as enhancers of *Src42A*

To identify genes that may interact with *Src42A* genetically, a search was made for fly mutants that enhance the eye phenotype induced by misexpression of the dominant-negative form of *Src42A* (*Src42A[KR]*) (Takahashi et al., 1996). As previously noted, eyes of flies heterozygous for a P[*Src42A[KR]*] insertion were almost entirely normal (Fig. 3A). Seven putative enhancer lines were obtained and E(7A-1), a line with the strongest enhancing activity (Fig. 3B), was selected for subsequent experiments.

Flies heterozygous for E(7A-1) were viable and not associated with any apparent morphological eye defects (data not shown), whereas E(7A-1) homozygotes were embryonic lethal. Complementation tests indicated the E(7A-1) lethal lesion to be present in 57B5-14 on the second chromosome, which contains *shotgun* (*shg*), a gene encoding E-cad (Tepass et al., 1996; Uemura et al., 1996). shg^{R64a} (a null allele) failed to complement E(7A-1). As with E(7A-1), shg^{R64a} enhanced the eye phenotype of flies heterozygous for P[*Src42A[KR]*] insertion (Fig. 3C). Virtually no E-cad signals could be found in *E(7A-1)* homozygous stage 13 embryos (Fig. 3D,E). Thus, we conclude that E(7A-1) harbors a lethal mutation in *shg* ($shg^{E(7A-1)}$) and that *shg* activity reduction enhances *Src42A* eye phenotypes.

Subsequent experiments indicated *shg* also capable of enhancing *Src42A* mutant phenotypes in various developmental contexts other than eye morphogenesis. shg^{R6} and $Src42A^{6-1}$ are hypomorphic alleles of *shg* and *Src42A*, respectively, (Niewiadomska et al., 1999) (this work) and dorsal closure of either $Src42A^{6-1}$ or shg^{R6} embryos appeared essentially normal (Fig. 3F,G). But most shg^{R6}; $Src42A^{6-1}$ embryos were associated with the dorsal open phenotype (Fig. 3I), indicating that *shg-Src42A* interactions are required for normal dorsal closure.

Src42A-shg interactions may also be involved in normal thorax closure in pupal stages. Fig. 3K-N shows defects in thorax closure in escapers and pharate adults of $Src42A^{6-1}$. Similar defects have been reported for $Src42A^{jp45}$ and classified into three classes (Tateno et al., 2000). $Src42A^{6-1}$ notum phenotypes were found considerably enhanced in the genetic background of shg^{g317}/+ (Fig. 3O). Two thirds of class 1 were converted to severer classes, while a fraction of class 3 was doubled. In some double mutant flies, right and left halves of the notum appeared completely separated from each other (class 4; Fig. 3N).

E-cad regulates cell-cell adhesion via homophilic association (Oda et al., 1994). Arm interacts directly with the cytoplasmic domain of E-cad and α-catenin. The latter is thought to associate with the actin network (Oda et al., 1993). Strong hypomorphic alleles of *arm* have defects in the dorsal closure (Grevengoed et al., 2001; McEwen et al., 2000), so we sought to determine whether *Src42A* interacts genetically with *arm* in dorsal closure and eye morphogenesis. As with embryos homozygous for $Src42A^{6-1}$, virtually all embryos heterozygous for arm^{YD35} (null allele) and those homozygous for $arm^{H8.6}$ (hypomorph) were normal in dorsal closure (Fig. 3H, data not shown). By contrast, most $Src42A^{26-1}$ embryos heterozygous for arm^{YD35} were associated with the dorsal open phenotype (Fig. 3J). $arm^{H8.6}$/+ eyes were normal in appearance, but *Src42A[KR]*/$arm^{H8.6}$ flies possessed rough eyes, as also noted for *Src42A[KR]/shg* (data not shown). It thus follows that *arm-Src* interactions are essential for normal dorsal closure and eye morphogenesis.

Requirements of Src activity for thick F-actin accumulation and adherens-junction maintenance at the leading edge

During early-mid stages of the dorsal closure, dorsal-most epidermal (DME) cells and epidermal cells located more ventrally elongate along the dorsoventral axis (Fig. 4A) and F-actin thickly accumulates at the leading edge (Fig. 5A). In the zippering stage, actin-based processes are essential for zippering epithelial sheets together (Jacinto et al., 2000). DME-cell elongation is associated with the redistribution of many proteins such as those involved in planar polarity and cytoskeleton (Kaltschmidt et al., 2002). Genetic experiments showed

interactions between *Src*, *shg* and *arm* to be involved in the dorsal closure and thus examination was made of temporal change in the locations of E-cad, Arm, Fas3 and F-actin during dorsal closure in *Src* and *shg* mutants as well as wild type.

In wild type, not only F-actin but also E-cad and Arm signals increased at the leading edge from 9 hours after egg laying (AEL; Fig. 4B and Fig. 5A) and polarized Fas3 expression and tubulin bundling occurred with dorsoventral elongation of dorsal epidermal cells (Fig. 4A) (Kaltschmidt et al., 2002). As stated above, no germ band retraction occurs in $Src42A^{26\text{-}1}$;$Src64^{P1}$ embryos (Fig. 2B5) and so examination was made of the effects of reduction in Src-activity on protein distribution at the leading edge of $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ and $Src42A^{26\text{-}1}$ embryos. In $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ embryos, DME-cell elongation and polarized deposition of Fas3 and tubulin bundling appeared to proceed normally (Fig. 4A). But, unlike wild-type embryos, $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ embryos exhibited significant reduction in E-cad and F-actin deposition at the leading edge at 11-12 hours AEL (Fig. 4B6,B8 and Fig. 5A4,A6). Arm signals appeared reduced throughout the entire membrane region, including the leading edge and accumulated in the cytoplasm. The leading edge of $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ embryos, initially smooth in appearance (Fig. 4B2), frequently kinked with partial DME-cell deformation from 10 hours AEL onwards (Fig. 4B4,B6,B8). Kinking of the actin cable at the zipper front is thought most likely due to lamellae traction (Jacinto et al., 2000), and, accordingly, Src activity reduction in $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ embryos may possibly give rise to defects in the cytoskeletal machinery that are essential for driving the dorsal closure. In $Src42A^{26\text{-}1}$ single mutant embryos, DME-cell elongation and the leading-edge structure appeared virtually normal (Fig. 4A9,B9 and Fig. 5B4)

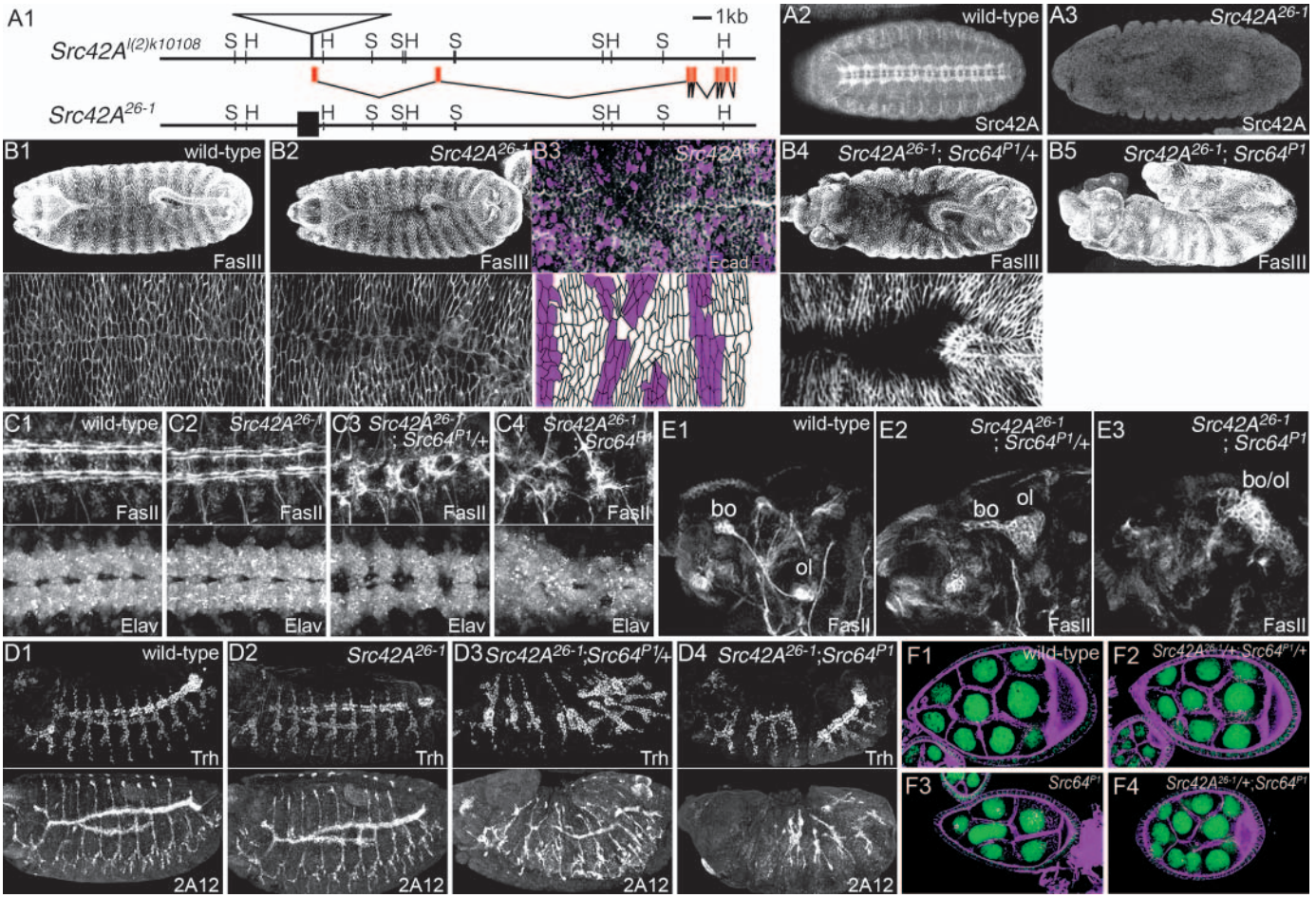

Fig. 2. Phenotypes of $Src42A^{26\text{-}1}$ mutants. (A1) Size and location of the $Src42A^{26\text{-}1}$ deletion are shown by the filled box. Triangle indicates P insertion; red boxes indicate exons; H, *Hin*dIII; S, *Sal*I. (A2,A3)Anti-Src42A antibody staining of wild-type (A2) and $Src42A^{26\text{-}1}$ (A3) embryos, indicating $Src42A^{26\text{-}1}$ a protein-null allele. (B1-B3) $Src42A^{26\text{-}1}$ embryos stained for Fas3 (B1,B2) or Engrailed (B3). These embryos were associated with slightly irregular midline (B1,B2; bottom). Staining for Engrailed (magenta) and E-cad (white) indicated cell-cell matching failure along the midline in these embryos (B3). Bottom panel provides an interpretation (magenta, Engrailed-expressing cells). (B4-F4) Functional redundancy of *Src42A* and *Src64* found in dorsal closure/germ-band retraction (B4,B5), CNS (C1-C4), trachea (D1-D4), visual system (E1-E3) and ovary (F1-F4). Anterior is towards the left. (B4) Dorsal view (top) and enlargement of the dorsal midline region (bottom) in $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ embryos. (B5) Lateral view of $Src42A^{26\text{-}1}$;$Src64^{P1}$ embryos. No germband retraction was observed. (C) CNS stained for Fas2 (top) or Elav (bottom). In *Src* double mutants, longitudinal axons were misrouted but neurons appeared not significantly reduced in number. (D) Embryos stained for Trachealess (Trh, top) and lumen (2A12, bottom). (E) Head regions stained for Fas2. Bolwig's organ (bo) fails to separate from the optic lobe (ol). (F) Ovaries stained with Rhodamine-phalloidin (magenta) and SYTOX (green).

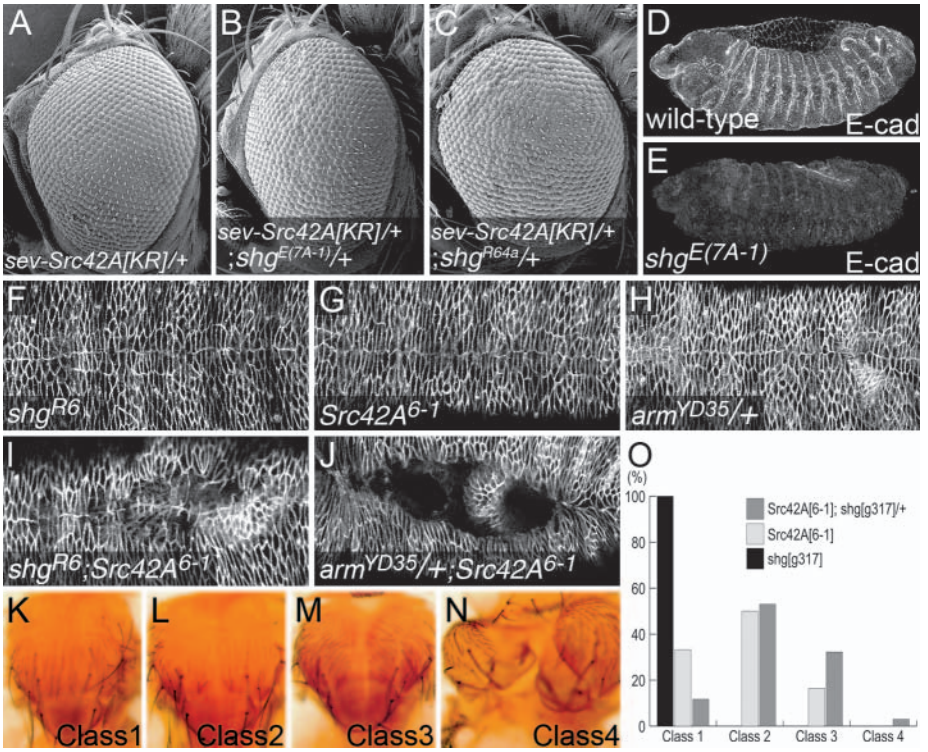

Fig. 3. Genetic interactions between *Src42A* and *shg/arm*. Anterior is leftwards in A-J. (A-C) Enhancement of the rough eye phenotype of *sev-Src42A[KR]/+* flies (A) by $shg^{E(7A-1)}$ (B) and shg^{R64a} (C). (D,E) Lateral views of stage 13 embryos of wild-type (D) and $shg^{E(7A-1)}$ (E) stained for E-cad, showing significant E-cad-signal reduction in $shg^{E(7A-1)}$. (F-J) Dorsal views of stage 15 embryos stained for Fas3, indicating dorsal open phenotype in double mutants. (K-N) Thorax-cleft phenotypes. Classification except for class 4 is based on Tateno et al. (Tateno et al., 2000). (O) Numerical data show that thorax-cleft phenotypes of $Src42A^{6-1}$ are enhanced by the introduction of a *shg* mutation, shg^{g317}.

but morphological defects could sometimes be seen along the zippered midline as mentioned above (see Fig. 2B3). Fig. 5B1-2 also shows that F-actin signals are also significantly reduced in shg^{R64a} mutant embryos.

It has been shown that no expression of *puckered* (*puc*) or *decapentaplegic* (*dpp*), positively regulated by JNK signaling (Goberdhan and Wilson, 1998) at the leading edge on *Tec29 Src42A* double mutants, suggesting that *Src42A* may act upstream of JNK signaling (Tateno et al., 2000). Puc is a negative regulator of JNK signaling and the absence of *puc* activity causes ventral expansion of the area of *dpp* expression normally restricted to DME cells (Martin-Blanco et al., 1997). Examination was thus made of *dpp* expression in *Src* mutants. *dpp* expression was monitored using nuclear *dpp-lacZ* signals

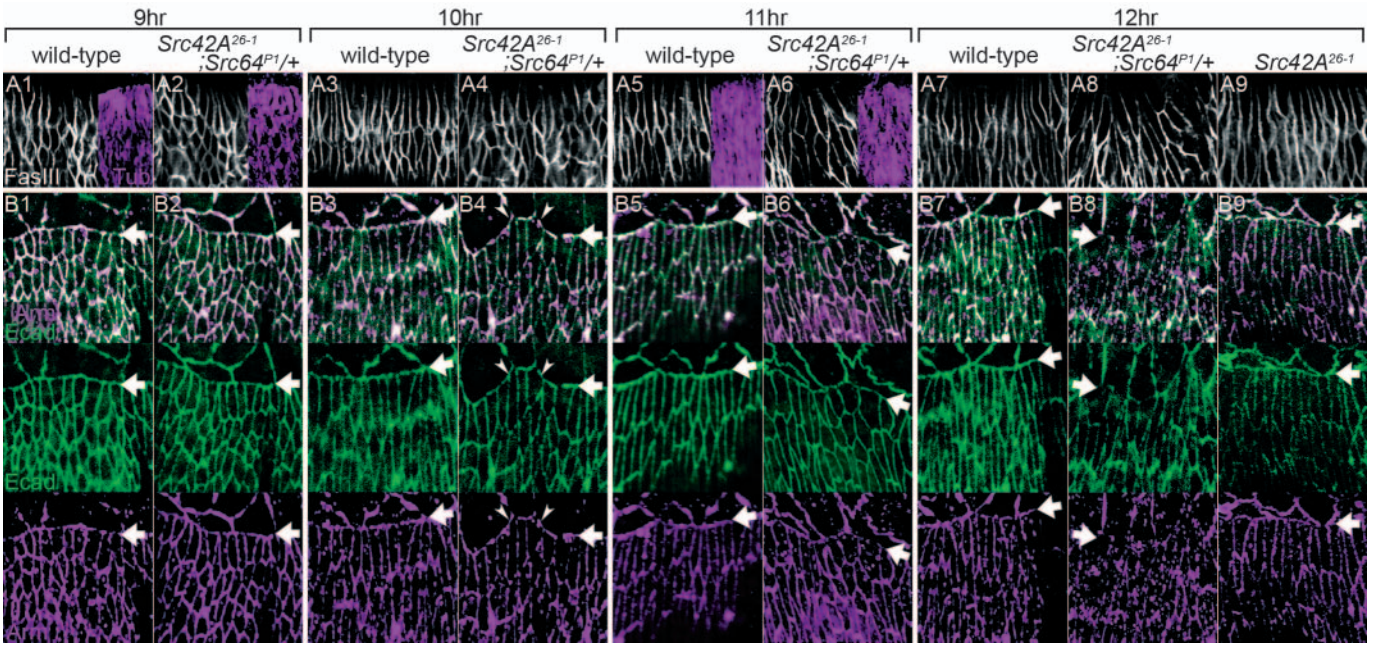

Fig. 4. Src-dependent temporal change in Fas3/tubulin (A1-A9) and E-cad/Arm (B1-B9) accumulation in DME-cells during dorsal closure. Embryos were collected at times indicated. Arrows indicate leading edge. (A) In wild-type, polarized expression of Fas3 and tubulin bundling and cell-elongation along the dorsoventral axis were observed at all times. (B) Leading edge was always smooth in appearance and leading edge signals of E-cad (green) and Arm (magenta) increased gradually 9-11 hours AEL. $Src42A^{26-1};Src64^{P1}/+$ embryos exhibited almost normal cell-shape change and signal deposition at 9 hours AEL (B2), but, at later stages, leading edge frequently kinked (arrowheads in B4) and simultaneously Arm and E-cad signals reduced significantly (B4,B6,B8). In B6 and B8, cytoplasmic Arm signals were found. $Src42A^{26-1}$ embryos were almost normal in cell-shape change and marker-protein deposition at the leading edge (A9,B9).

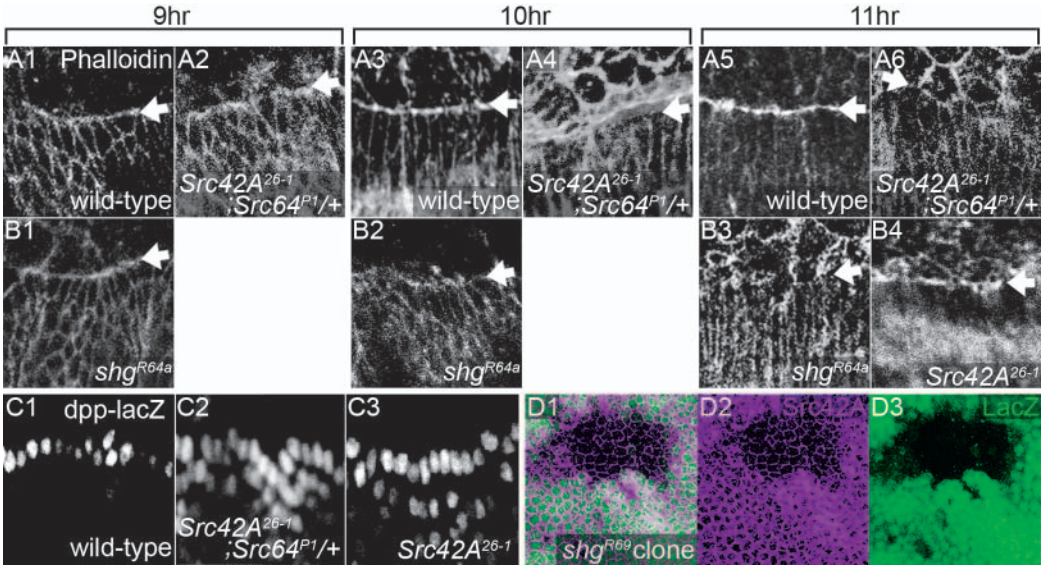

Fig. 5. F-actin accumulation in DME-cells during dorsal closure. Embryos were collected at times indicated. Arrows indicate the leading edge. (A) In wild type, leading edge signals of F-actin increased gradually during 9-11 hours AEL. *Src42A*$^{26\text{-}1}$*;Src64*^{P1}/+ embryos exhibited almost normal F-actin deposition at 9 hours AEL, but, at later stages, leading edge F-actin was reduced significantly. *Src42A*$^{26\text{-}1}$ embryos were almost normal in F-actin deposition at the leading edge (B4). In *shg*^{R64a} mutants (B1-B3), thick accumulation of F-actin at leading edge disappeared at 10 hours AEL. (C1-C3) Change in *dpp* expression at leading edge. *dpp* expression, which is under the control of the JNK signaling (Goberdhan and Wilson, 1998), was monitored using nuclear enhancer trap (*dpp-lacZ*). In *Src* mutants, *dpp* expression has ventrally expanded as in the case of *puc* mutants (Martin-Blanco et al., 1997). (D1-D3) Effects of *shg* activity on Src42A distribution. *shg*^{R69} clone on pupal wing disc was marked by the absence of *lacZ* signals (green). In the *shg*^{R69} clone, Src42A signals (magenta) in plasma membrane were significantly reduced. Merged picture is on the left.

(Jiang and Struhl, 1995). Unlike *Tec29 Src42A* mutants, ventrally expanded *dpp* expression was found in all *Src* mutants (Fig. 5C1-3), indicating that JNK signaling was not completely suppressed in *Src42A*$^{26\text{-}1}$;*Src64*^{P1}/+ and *Src42A*$^{26\text{-}1}$ mutant embryos.

In epithelial cells, membranous Src42A is colocalized with E-cad (Fig. 1O) and, consequently, *shg* activity may be required for proper plasma membrane localization of Src42A. To confirm this point, *shg*^{R69} clones were generated in pupal wing discs and Src42A localization was examined for any change by anti-Src42A antibody staining (Fig. 5D1-D3). Membranous Src42A signals in *shg* mutant clones were found to be reduced significantly in a cell-autonomous fashion, indicating that *shg* is essential for proper plasma membrane localization of a certain region of Src42A.

Taken together, our results indicate that the leading-edge adherens junction containing E-cad, Arm and actin may serve as a cytoskeletal and/or regulatory machinery for properly driving the dorsal closure, and that interactions between *Src42A*, *shg* and *arm* would be essential for membrane localization of their own protein products.

Src-activity-dependent induction of cell migration and cell-shape change

To further clarify Src function, activated (*Src42A[YF]*), dominant-negative (*Src42A[KR]*) and wild-type (*Src42A[WT]*) forms of *Src42A* were driven by *pnr*-GAL4 to determine any change in Arm, E-cad or Src42A signals (Fig. 6A1-D3). Immunostaining of embryos collected at 10-14 hours AEL showed that, as with wild type (control; Fig. 6A1-A2), nearly all Arm and E-cad signals localize in the plasma membrane when kinase-inactive *Src42A[KR]* is driven (Fig. 6B1-B3), while considerable cytoplasmic E-cad and Arm signals are evident in cells overexpressing *Src42A[WT]* or *[YF]* (Fig. 6C1-3,D1-3). It may thus follow that activated Src stimulates cytosolic Arm stabilization and/or *arm* expression. Alternatively, Src42A may be involved in regulating possible cadherin endocytosis.

Overexpression of activated *Src42A* may also cause change in cell morphology. As shown in Fig. 6A1-D1, forced expression of UAS-*Src42A[WT]* and *[YF]* prevented dorsal epithelial cells from elongating normally. Occasionally, cells that strongly expressed Src42A and were separated from the amnioserosa or dorsal epidermis plane could be found in embryos transformed with *Src42A[YF]* (Fig. 6E-G). These cells were frequently associated with the expression of Cll (Kojima et al., 2005) or Fas3, which are maker proteins for amnioserosa and epidermis, respectively (Fig. 6H-K) (Jagla et al., 2001; Kaltschmidt et al., 2002; Kojima et al., 2005), but not with TUNEL signals (Booth et al., 2000) at least up to the end of stage 13 (Fig. 6L,M,O,P), suggesting that they are live cells dissociated from amnioserosa or dorsal epidermis because of elevated Src activity. Most released cells degenerated at stage 16 via apoptosis (Fig. 6N,Q). We conclude that *Src42A* is essential for proper cell migration and cell-shape regulation.

Physical binding of Src42A to Arm through kinase-domain/Arm-repeat interactions

To determine the molecular basis for genetic interactions between *Src42A*, *shg* and *arm*, study was made as to whether or not protein products of these genes come together to form complexes within cells was examined using fractionated embryonic extracts. Membrane and cytosolic fractions were prepared from wild-type embryos and embryos with *pnr*-GAL4-dependent forced expression of either UAS-*Src42A[WT]*, *[KR]* or *[YF]*. Here, we describe only endogenous interactions in wild-type embryos, whereas physical interactions in embryos with forced *Src* expression are described in the next section.

Membrane and cytosolic fractions of wild-type embryos were treated with anti-Arm or anti-E-cad antibodies and the

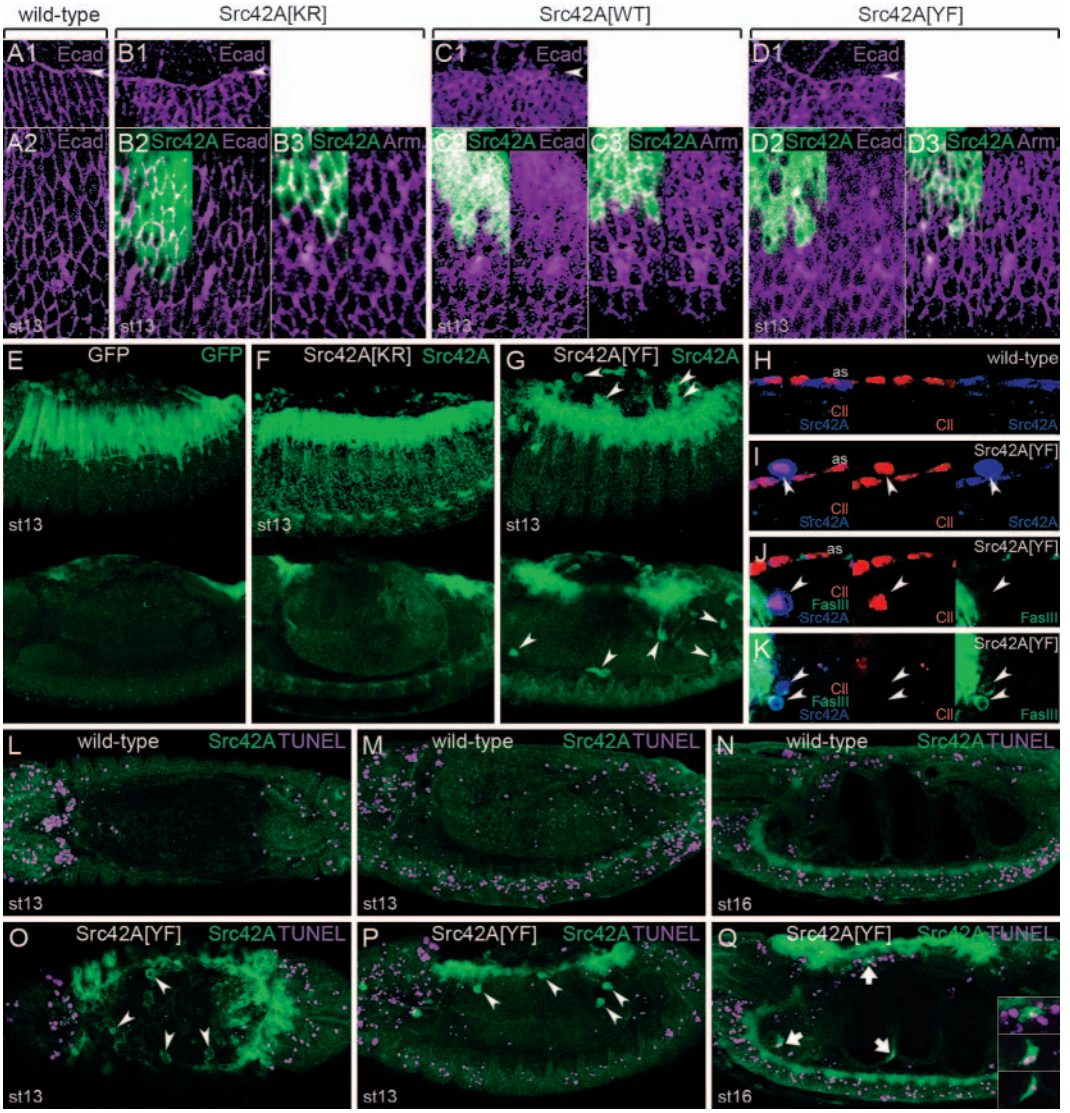

Fig. 6. Effects of Src42A[KR] (B1-B3), Src42A[WT] (C1-C3) or Src42A[YF] (D1-D3) on cell-shape and Arm/E-cad distribution at stage 13 and possible release of *Src*-overexpressing cells from amnioserosa and dorsal epidermis (E-Q). *pnr*-GAL4 was used as driver for forced expression of *Src42A* genes. Green, Src42A; magenta, E-cad or Arm. (A1,A2) Wild type. Anterior is leftwards and dorsal upwards. Unlike Src42A[KR] (B1-B3), Src42A[WT] and Src42A[YF] expression caused significant change in morphology and increased cytoplasmic Arm and E-cad signals (B1-D3). (E-G) Arrowheads indicate Src-overexpressing isolated cells in Src42A[YF] embryos. (H-K) Cll (red) and Fas3 (green) are markers for amnioserosa (Jagla et al., 2001) and epidermis, respectively. Blue, Src42A. Most Src-overexpressing isolated cells (arrowheads in I-K) exhibited Cll (H-J) or Fas3 (K). (L-Q) Src-overexpressing isolated cells were TUNEL-stained (magenta) at stage 16 (N, arrows in Q) but not stage 13 (L,M, arrowheads in O,P). Green, Src42A. Insets in Q show enlarged TUNEL-positive isolated cells.

resultant immunoprecipitates were analyzed by SDS-PAGE and subsequent western blotting (Fig. 7B-C). Any appreciable Src42A/Arm signals were detected in the E-cad immunoprecipitates of untransfected S2 cells, which expresses only a low level of E-cad (Fig. 7A, lane 1). Fig. 7B also shows that unrelated anti-Fas3 antibody gave no Src42A/Arm signals.

As shown in Fig. 7C, parts e-g (lane 1), not only Arm but also Src42A and E-cad signals were detected in anti-Arm antibody immunoprecipitates obtained from the membrane fraction. Membranous Src42A signals were also co-precipitated by anti-E-cad antibody treatment (Fig. 7C, part h, lane 1). By contrast, there were no appreciable signals of E-cad on treating the cytosolic fraction with anti-Arm antibody (Fig. 7C, part f, lane 5). In the cytosolic fraction, Src42A signals co-precipitated with Arm appeared much less prominent than those in the membrane fraction (Fig. 7B, lanes 2, 4; Fig. 7C, part g, lanes 1,5). Accordingly, significant fraction of membranous Arm, a core component of the putative adherens junction, may be considered to form a complex directly or indirectly with E-cad and Src42A as well.

Arm protein possesses 13 repeats referred to as Arm repeats, which provide binding sites for many Arm/β-catenin-binding proteins (Provost and Rimm, 1999). To determine whether Src42A binds to Arm directly and if so which part of Src42A is responsible for the Src-Arm interaction, pull-down assay was carried out (Fig. 7D). Strong GST-Arm-repeat (GST-Arm^{R}) signals were recognized in the lanes for MBP-Src42A[Kinase] and the 14 amino acid autophosphorylation site-containing peptide (Fig. 7D, lanes 6,8). But no signals could be found in lanes for MBP-Src42A[SH3SH2], MBP-Src42A[(Kinase)-(391/404)] or MBP (Fig. 7D). Src42A is thus shown to bind to Arm through interaction of the 14 amino acid kinase domain peptide with Arm repeats.

Requirements of Src for Arm phosphorylation

In vertebrates, tyrosine phosphorylation has been shown to cause the binding of β-catenin to E-cad to diminish significantly. Interactions between β- and α-catenin may also be negatively regulated by tyrosine phosphorylation (reviewed by Lilien et al., 2002). We therefore studies whether Arm phosphorylation requires Src activity. Arm was overexpressed

in *Drosophila* S2 cells and RNAi was carried out to clarify any *Src42A* and/or *Src64* involvement in Arm tyrosine-phosphorylation (Fig. 7E). dsRNAs used specifically abolished protein expression of the corresponding target genes. Change in the degree of Arm tyrosine residue phosphorylation was monitored with Arm western blotting of anti-pTyr antibody immunoprecipitates of whole or E-cad-free cell extracts. The levels of Tyr-phosphorylated Arm were reduced by transfection

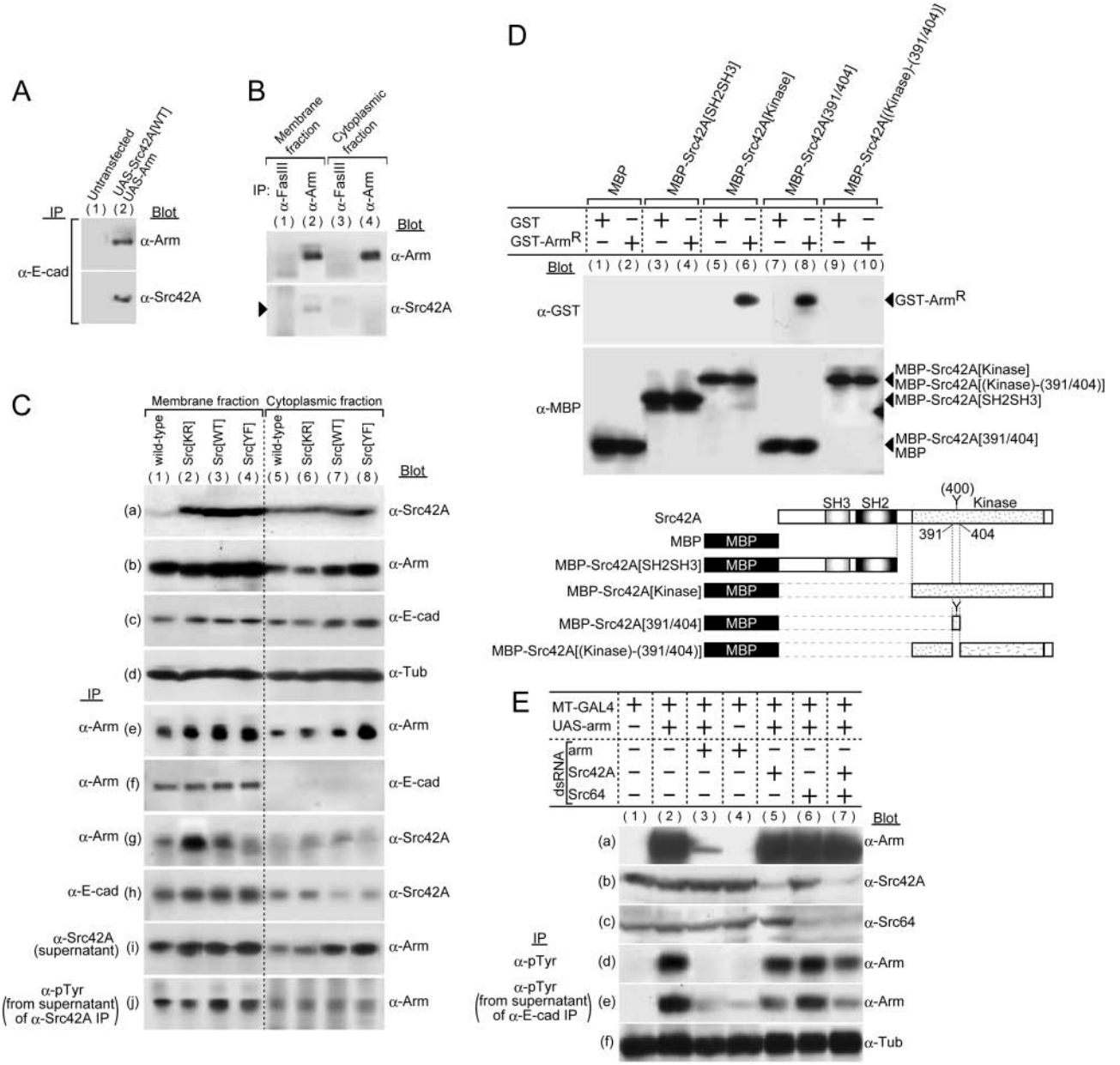

Fig. 7. Src42A/E-cad/Arm ternary complex formation and Src-dependent Arm tyrosine-phosphorylation. (A) Arm/Src42A signals in E-cad immunoprecipitates of untransfected and *Src42A*/*arm*-transfected S2 cells. (B) Comparison of Arm/Src42A signals in cytosolic and membranous anti-Fas3 and anti-Arm immunoprecipitates of wild-type embryos. Identical amounts of membrane and cytosolic Arm were precipitated and examined. (C) Immunoprecipitation of membranous and cytosolic fractions of wild-type, Src42A[KR], Src42A[WT] and Src42A[YF] embryos. Same volumes of membrane and cytosolic fractions were analyzed. (a-d) Western blots of fractionated extracts. Tubulin is used as a control. (e-g) Arm, E-cad and Src42A blots of Anti-Arm antibody immunoprecipitates. (h) Src42A blots of anti-E-cad-antibody immunoprecipitates. (i,j) Arm blots of the supernatant of anti-Src42A antibody immunoprecipitates with (j) or without (i) anti-pTyr-antibody treatment. (D) Pull-down assay. Src42A was dissected as shown in the lower margin and expressed as MBP fusions. Y indicates the autophosphorylation site. GST-Arm-repeat signals were detected only in lanes 6 and 8, indicating Arm repeats to bind to the 14 amino acid, autophosphorylation-site-containing Src42A[Kinase] fragment. Lane 10 may indicate that the kinase domain other than the autophosphorylation peptide is unrelated to Arm/Src42A interactions. Input protein signals are shown in lower half as anti-MBP antibody signals. (E) Src dependency of Arm tyrosine phosphorylation. Western blots of extracts from S2 cells transfected with various combinations of metallothionein (MT)-GAL4 and UAS-*arm*, and dsRNAs indicated are shown (a-f). Signals for whole cell (d) and E-cad-free (e) tyrosine-phosphorylated Arm significantly reduced by Src42A RNAi (compare lane 2 with lane 5).

with *Src64* and/or *Src42A* dsRNAs (Fig. 7E, parts d,e, lanes 2,5-7), indicating that redundant Src function is required for Arm phosphorylation.

Consistent with the present findings on cytological accumulation of cytoplasmic E-cad and Arm signals in cells overexpressing Src42A[WT] or [YF] (Fig. 6C1-3,D1-3), increased signals of Arm and E-cad were noted in cytosolic fractions obtained from cells with forced expression of *Src42A[WT]* or *Src42A[YF]* when using α-tubulin or Src42A as the control (Fig. 7C, parts a-d, lanes 5-8). Similar *Src42A* activity-dependent increase in cytosolic Arm signals was observed in anti-Arm precipitates and the supernatant fluid of anti-Src42A precipitates (Fig. 7C, parts e,i, lanes 5-8). Such increase may possibly be due to Arm tyrosine residue phosphorylation. However, we consider that cytosolic Arm accumulation in cells with forced expression of *Src42A[WT*] or *Src42A[YF]* may not necessarily arise from the tyrosine phosphorylation of Arm. Indeed, as shown in Fig. 7C, part j (lanes 5-8), any *Src42A*-activity-dependent tyrosine phosphorylation appeared absent from Src42A-free Arm in the cytosolic fraction. Possibly, Src42A-dependent tyrosine phosphorylation activates some unknown factor responsible for cytosolic Arm stabilization, but not Arm itself.

Discussion

The results of this study clearly demonstrate the redundant function of *Src42A* and *Src64* to be indispensable in numerous aspects of *Drosophila* development. Though Src42A is distributed over the entire plasma membrane of all cells, its signal distribution is not uniform. Two major types of Src42A deposition in the membrane could be clearly recognized (Fig. 1).

In ectodermal cells, strong Src42A signals in apical or apicolateral regions were always associated with strong E-cad signals (Fig. 1J). E-cad is a core component of the adherens junction that is responsible for cell-cell adhesion (reviewed by Takeichi, 1990) and, hence, most, if not all, E-cad-associated membranous Src42A are probably related to adherens junction-dependent cell-cell adhesion.

A considerable fraction of ectodermal cells were also found associated with the second type of basal Src42A free of E-cad (Fig. 1L,N,R). E-cad-free Src42A was localized on the ectoderm/mesoderm interface and eliminated from ectodermal cells, which had evaginated or invaginated without mesoderm association (Fig. 1N,P,R). The extracellular matrix (ECM) comprises several groups of secreted proteins such as integrin ligands. During embryogenesis, different cell layers become properly connected, most probably via cell adhesion to ECM (Yurchenco, 1994). E-cad-free Src42A may thus be related to integrin-mediated cell-matrix adhesion. Cell-ECM adhesion may not be restricted to the interface between ectodermal and mesodermal cell layers. Strong Src42A signals have actually been found present on the interface between mesodermal and endodermal cell layers.

Requirements of Src incorporated into putative adherens junction for *Drosophila* development

JNK signaling, which includes *hemipterous* (*hep*) and *bsk*, is essential for dorsal closure of the embryonic epidermis in *Drosophila* (reviewed by Goberdhan and Wilson, 1998). Based on examination of *Tec29 Src42A* mutant phenotypes, it was considered that *Src42A* may act upstream of *bsk* (Tateno et al., 2000). Consistently, our study showed that, as with JNK signaling genes (Kaltschmidt et al., 2002), *Src* is required not only for thick F-actin accumulation at the leading edge (Fig. 5A) but proper cell-cell matching along the midline seam as well (Fig. 2B3).

In vertebrates, JNK is considered to be situated downstream of Src in integrin signaling (Oktay et al., 1999; Schlaepfer et al., 1999). Our genetic experiments (Fig. 3) would indicate that interactions between *Src* and *arm*/*shg*, genes encoding the core components of the adherens junction are essential for JNK signaling regulation required for dorsal closure. A pull-down assay (Fig. 7D) also showed that Src protein is capable of directly binding to Arm. Both putative adherens-junction Src and integrin-associated Src thus would appear involved in the regulation of JNK signaling.

The adherens junction is necessary for cell-cell adhesion (reviewed by Takeichi, 1990) and thick F-actin accumulation occurs at the level of the adherens junction at the leading edge (Kaltschmidt et al., 2002). As E-cad and Arm signals along with actin signals were reduced significantly at the leading edge in $Src42A^{26\text{-}1}$;$Src64^{P1}$/+ embryos (Fig. 4B8) and the leading edge of the mutants was significantly kinked (Fig. 4B4,B6,B8), the absence of Src protein from the adherens junction may possibly result in destruction of structural integrity, implying that adherens junction is also involved in dorsal closure regulation in a structural way.

Dorsal closure and CNS defects similar to those in *Src* mutants were previously observed in *abl* mutants (Grevengoed et al., 2001). In vertebrates, Abl is tyrosine-phosphorylated with Src (Plattner et al., 1999) and is capable of interacting with δ-catenin, an E-cad-binding protein (Lu et al., 2002). Abl may thus function as well downstream of Src signaling in *Drosophila*. Germ-band retraction and possibly too, head involution, both of which require Src activity (this work), may be regulated by the two above distinct Src functions. α1,2-laminin and αPS3βPS integrin have clearly shown to be essential for spreading a small group of amnioserosa epithelium cells over the tail end of the germ band during germ-band retraction (Schoeck and Perrimon, 2003). Our unpublished data indicate that *shg* activity is essential for normal germ-band retraction and head involution.

Src-dependent dynamical regulation of E-cad-dependent cell-cell adhesion may also necessary for visual system formation. E-cad overexpression or elimination of EGFR activity have been shown to render optic placode cells incapable of invaginating and prevent the separation of Bolwig's organ precursors from the optic lobe (Dumstrei et al., 2002). Virtually identical phenotypes were induced by loss of *Src* activity (this work), suggesting involvement of at least the adherens junction Src in larval visual system formation and that Src should function either upstream or downstream of EGFR signaling.

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