Regulation of cell–cell contacts in developing *Drosophila* eyes by *Dsrc41*, a new, close relative of vertebrate c-src

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In *Drosophila*, *Dsrc64* is considered a unique ortholog of the vertebrate c-src; however, we show evidence to the contrary. The closest relative of vertebrate c-src so far found in *Drosophila* is not *Dsrc64*, but *Dsrc41*, a gene identified for the first time here. In contrast to *Dsrc64*, overexpression of wild-type *Dsrc41* caused little or no appreciable phenotypic change in *Drosophila*. Both gain-of-function and dominant-negative mutations of *Dsrc41* caused the formation of supernumerary R7-type neurons, suppressible by one-dose reduction of *boss*, *sev*, *Ras1*, or other genes involved in the Sev pathway. Dominant-negative mutant phenotypes were suppressed and enhanced, respectively, by increasing and decreasing the copy number of wild-type *Dsrc41*. Colocalization of *Dsrc41* protein, actin fibers and DE-cadherin, and *Dsrc41*-dependent disorganization of actin fibers and putative adherens junctions in precluster cells suggested that *Dsrc41* may be involved in the regulation of cytoskeleton organization and cell–cell contacts in developing ommatidia.

[Key Words: Protein tyrosine kinase; *Drosophila*; src; *Dsrc41*; cadherin; boss]

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The Src tyrosine kinase family in vertebrates consists of nine members (for review, see Cooper and Howell 1993), at least three of which—*src*, *yes*, and *fyn*—are widely expressed in a variety of cells including those in the neural ectoderm and developing cerebellum (Cotton and Brugge 1983; Fuhs et al. 1985; Manness et al. 1986; Zhao et al. 1990). These tyrosine kinases may participate in developmental processes such as neural and hematopoietic cell differentiation (Molina et al. 1992; Yagi et al. 1993). Studies using knockout mice suggest that compensatory interactions occur between Src family tyrosine kinases. Only a restricted phenotype, or no overt dysfunctional consequences is observed in a loss-of-function mutant of *src*, *yes*, and *fyn*, whereas most *src/fyn* or *src/yes* double mutants die perinatally and a considerable number of *fyn/yes* double mutants undergo degenerative renal damage with consequent diffuse segmental glomerulosclerosis (Stein et al. 1994).

Elimination of the conserved tyrosine residue in the carboxyl terminus endows Src family tyrosine kinases with transforming activity. This activity is thought to be induced by derepression of tyrosine kinase activity (Cartwright et al. 1987), which is normally repressed via stoichiometric phosphorylation of the carboxy-terminal ty-

rosine residue by tyrosine kinase kinases such as Csk. Individuals with homozygous deficiency of *csk* are embryonic lethal (Nada et al. 1993; Imamoto and Soriano 1993). Because of this repression mechanism, the overexpression of c-src in fibroblasts has no significant effect on cell growth or morphology, nor overall abundance of tyrosine-phosphorylated proteins (Shalloway et al. 1984; Iba et al. 1985).

In *Drosophila*, three *src*-related genes, *Dash* (Henkemeyer et al. 1988), *Dsrc29* (Gregory et al. 1987), and *Dsrc64* (Simon et al. 1985), have been identified using a v-src fragment as a probe. Only the polypeptide encoded by *Dsrc64* (*Dsrc64*) resembles, in its entire structure, vertebrate Src and is presumed to belong to the Src family (Simon et al. 1985). *Dash* may be a counterpart of vertebrate *abl* (Henkemeyer et al. 1988), whereas *Dsrc29* is suggested to be in the same family as mammalian tyrosine kinase genes *itk* and *tec* (Tsukada et al. 1993). Recently, however, Kussick et al. (1993) showed *Dsrc64* to have properties considerably different from those of vertebrate c-Src. In contrast to vertebrate c-Src overexpressed in fibroblasts (Iba et al. 1985), wild-type *Dsrc64* overexpressed in *Drosophila* was phosphorylated not only at the carboxy-terminal tyrosine residue but at major in vitro autophosphorylation sites as well (Kussick and Cooper 1992), causing embryonic lethality or abnormal differentiation (Kussick et al. 1993). Thus possibly,

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carboxy-terminal tyrosine phosphorylation of Dsrc64 is substoichiometric (Kussick and Cooper 1992).

Here, we show isolation and characterization of *Dsrc41*, a new *src*-related gene in *Drosophila*. Nucleotide sequence analysis and analysis of phenotypes of gain-of-function and dominant-negative mutations in developing retina and embryos suggested that *Dsrc41* is the closest relative of vertebrate *c-src* found so far in *Drosophila* and may play an essential role in cytoskeleton organization and cell-cell contacts in development.

Results

Molecular cloning of *Dsrc41*, the closest relative of vertebrate *c-src* found so far in *Drosophila*

dtk-5 is a PCR fragment encoding a partial tyrosine kinase sequence (Shishido et al. 1991). During the course of searching *dtk5*-related genes in *Drosophila*, we discovered two λ clones, λ gfk1 and λ gfk2 (Fig. 1A), which together encode the putative polypeptide showing much greater similarity in amino acid sequence to vertebrate *c-Src* than *Dsrc64* (see Fig. 2B). As its cDNA hybridized at 41B or 41C on the second chromosome (data not shown), we refer to the gene hereafter as *Dsrc41*.

Nucleotide sequences of the longest cDNA and relevant genomic DNA fragments were determined (Fig. 2A). The longest cDNA was 2.9 kb in length, which would agree well with the 3-kb RNA observed in Northern blots (Fig. 1B). *Dsrc41* contains 9 exons that would code for a polypeptide of 517 amino acid residues (Figs. 1A and 2A). The *Dsrc41* gene product (*Dsrc41*) resembled the vertebrate *Src* family kinase consensus over the entire SH1 (catalytic), SH2, and SH3 domains, the amino-terminal myristylation site, and the carboxy-terminal tail (Fig. 2A,B).

As shown in Figure 2B, the kinase domain of *Dsrc41* was much more similar in amino acid sequence to those of vertebrate *Src* members than *Dsrc64*. On average 173 amino acids were conserved between *Dsrc41* and five vertebrate *Src* members (*Src*, *Yes*, *Yrk*, *Fgr*, and *Fyn*), whereas only 148 amino acids were invariant between *Dsrc41* and *Dsrc64*. Similarity in amino acid sequence between *Dsrc41* and vertebrate *Src* members was also evident in SH3 and the carboxy-terminal tail (Fig. 2B), making possible unequivocal assignment of Tyr-511 of *Dsrc41* to *c-Src* Tyr-527, whose phosphorylation is essential for the regulation of kinase activity within cells (Cartwright et al. 1987). Structural resemblance between

Figure 1. (A) Genomic and cDNA structures of *Dsrc41*. (λ gfk1 and λ gfk2) Genomic clones; (λ cfk) the longest cDNA clone. (Open boxes) Presumed coding regions. (Bar A) Hybridization probe. (H) *Hind*III; (S) *Sal*I. (B) Northern blotting of *Dsrc41* RNA. (Lanes 1–4) RNA extracted from embryos, total larvae, total pupae, and adults (males and females), respectively. (Arrowhead) 3-kb *Dsrc41* transcript. (C) Southern analysis of *Dsrc41*. Canton-S adult DNA was terminally digested with *Eco*RI (lane 1) and *Hind*III (lane 2). Probe, fragment A in A.

vertebrate *c-src* and *Dsrc41* can be extended further to exon–exon junctions (Fig. 2E). In contrast, no correlation in exon–exon junctions was detected between *Dsrc64* and *c-src*. *Dsrc64* thus likely forms its own group distantly related to *Dsrc41* and vertebrate *src* family members (Fig. 2C).

Drosophila may contain no close relative of *Dsrc41*, because as shown in Figure 1C, no band other than those for *Dsrc41* could be detected by hybridization under less stringent conditions.

Temporal and spatial expression of *Dsrc41* RNA

Poly(A)⁺ RNA extracted from embryos, larvae, pupae, and adults was examined by Northern blotting (Fig. 1B). A single transcript, 3 kb long, was found in both embryos and pupae. Its spatial patterns in embryos and imaginal discs were also examined, as shown in Figure 3. In very early embryos (A), little or no *Dsrc41* RNA was detected, suggesting the level of its maternal expression to be quite low, if any. *Dsrc41* RNA was first discernible at stages 2 or 3 (Campos-Ortega and Hartenstein 1985). At

Figure 2. (A) Nucleotide sequence of the *Dsrc41* gene and the deduced amino acid sequence of *Dsrc41*. Lowercase letters show the 5'-genomic flanking sequence. Overlines *a* and *b* indicate putative TATA-like sequence and the RNA start, respectively. (▼) Positions of introns. SH3, SH2, and SH1 domains are boxed. The lysine residue for ATP binding and two regulatory tyrosine residues are circled. The GSDB/DBJ/EMBL/NCBI accession no. for the *Dsrc41* sequence is D42125. (B) Sequence homology in SH1. Residues identical to the counterparts of *Dsrc41* are shaded. Amino acid sequences of *c-Src* (chicken), *Yes* (human), and *Dsrc64* are from Hanks et al. (1988). Broken underlines indicate the SH1 domain. (C) A phylogenetic tree of the *src* family. The location of *Dsrc41*, which was calculated based on amino acid sequence homology, was superimposed on the phylogenetic tree (Hanks et al. 1988). Thus, the length and exact branching position of the broken line are inaccurate. (D) Structures of various P-based *src* constructs. (E) Conserved exon–exon junctions.

D

vermilion hsp promoter K276 Y511 poly A

sE²-Dsrc41^{WT}

K F

sE-Dsrc41^{YF}

R Y

sE²-Dsrc41^{KR}

R F

sE²-Dsrc41^{KRYF}

K F

sE²-Dsrc41^{YF}/lacZ

1647

Figure 3. Spatial and temporal expression of *Dsrc41* RNA. In embryos, anterior is to the left. (A) Very early stage. (B) Syncytial blastoderm. (C) Cellular blastoderm. (D) Germ band-extended stage. (E) Stage 13. The arrowhead indicates the visceral mesoderm. (F,G) Stages 15–16. Arrowheads *b*, *h*, and *v* indicate brain, hindgut, and ventral ganglions, respectively. The small arrowheads in *G* indicate anal pads. (H) *Dsrc41*-deficient stage-16 embryo homozygous for the *In(2R)bw^{De21}Cy^R* chromosome. No *Dsrc41* RNA could be detected. (I) A heat-shocked embryo homozygous for the *Dsrc41*^{WT} insertion (WT-A). (J,K) *sE-Dsrc41*^{Yf} and wild-type embryos treated with heat shock at 37°C for 30 min at 4–7 hr of development and then cultured at 25°C until 13–16 hr of development. CNS was stained with anti-HRP. Brackets show CNS shortening to be blocked in *J*. (L–P) Third-instar wing disc (L), CNS (M), leg disc (N), eye–antennal disc (O), and an enlargement of O (P). Anterior is at the top. (Arrowhead) Morphogenetic furrow.

the cellular blastoderm stage, it was expressed weakly in virtually all cells (C). Uniform but somewhat stronger expression persisted until the end of germ-band retraction (D). During stages 13–16, relatively high expression was recognized in visceral mesoderm (E), hindgut, brain, anal pads, and ventral ganglions (F,G). No signal of *Dsrc41* RNA was detected in embryos homozygous for a deficiency of *Dsrc41* (H). In third-instar larvae, *Dsrc41* RNA expression was still strong in the central nervous system (CNS) (M). Almost all cells expressed *Dsrc41* RNA in wing (L), leg (N), and eye–antenna (O,P) discs. In contrast, *Dsrc64* expression in eye discs has been reported to be restricted to photoreceptor precursors posterior to the morphogenetic furrow (Kussick et al. 1993).

In vivo functions of *Dsrc41* and vertebrate *c-Src* are similarly regulated by the carboxy-terminal conserved tyrosine residue

Overexpressed *c-src* in fibroblasts induces no appreciable phenotypic change (Shalloway et al. 1984), whereas elimination of the carboxy-terminal tyrosine residue results in activation of intrinsic catalytic activity and malignant transformation (Cartwright et al. 1987). Using *Drosophila* compound eye and embryonic systems, we examined effects of the overexpression of *Dsrc41* with or without the conserved carboxy-terminal tyrosine residue.

DNA fragments containing either wild-type *Dsrc41*

(*Dsrc41*⁺) or a constitutively active mutant containing a Tyr → Phe substitution at position 511 (*Dsrc41*^{YF}) were subcloned into P-based expression vectors possessing one or two *sevenless* (*sev*) enhancers (*sE* or *sE*²) associated with the *hsp70* promoter (Fig. 2D); *sE*² is more active than *sE* (Bowtell et al. 1991). Thus, the expression of the inserted *Dsrc41* gene is bipartite; the *hsp70* promoter enables heat shock-inducible, ubiquitous expression, whereas *sev* enhancers drive high expression in a limited species of ommatidial cells. Using these constructs, three and two transformant lines containing *sE*²-*Dsrc41*⁺ (WT-A, WT-B, and WT-C) and *sE*-*Dsrc41*^{YF} (YF-A and YF-B), respectively, were obtained. In situ hybridization showed these transgenes to be overexpressed in the region posterior to the morphogenetic furrow in eye discs and heat-shocked embryos (Fig. 3I).

As with flies with activated *Sev* tyrosine kinase (*Sev*^{S11}; Basler et al. 1991), *Dsrc41*^{YF} flies exhibited rough eyes (Fig. 4B) associated with extra R7-type neurons resistant to the [outer rhabdomeres absent] (*ora*) mutation (Stark and Sapp 1987) (Fig. 5B,E). An ommatidium heterozygous for the *sE*-*Dsrc41*^{YF} transgene insertion (YF-A) contained two to three R7-type neurons (Table 1), and the value increased with increasing copy of the inserted transgene (data not shown). In contrast, no appreciable

Figure 4. Scanning electron micrographs of compound eyes. (A) Wild type. (B) Heterozygote for *sE*-*Dsrc41*^{YF} insertion (YF-A). (C) Homozygote for *sE*²-*Dsrc41*⁺ insertion (WT-A). (D) Heterozygote for *sE*²-*Dsrc41*^{KR} insertion (KR-A). (E) Double heterozygote for *sE*²-*Dsrc41*^{KR} and *sE*-*Dsrc41*⁺ insertions. (F) Double heterozygote for *sE*²-*Dsrc41*^{KR} insertion and a *Dsrc41* deficiency.

Figure 5. Tangential sections at the R7 level. (A) Wild type. (B) A transformant line (YF-A) heterozygous for the *sE*-*Dsrc41*^{YF} insertion. (C) A transformant (WT-A) homozygous for the *sE*²-*Dsrc41*⁺ insertion. (D) Outer photoreceptor rhabdomeres are absent from the *ora* mutant. (E) Supernumerary rhabdomeres in YF-B are stable against the *ora* background. (F-H) Fly strains doubly heterozygous for *sE*-*Dsrc41*^{YF} insertion (YF-A) and one of a mutation of sevenless pathway components. (F) *Ras1*^{e2F}. (G) *boss*¹. (H) *sev*^{dl2}. (I) Heterozygote for *sE*²-*Dsrc41*^{KR} insertion (KR-A). (J,K) *sE*²-*Dsrc41*^{KR} heterozygous for *sE*²-*Dsrc41*⁺ insertions (J) or *Ras1*^{e2F} (K). (L) *sE*²-*Dsrc41*^{KR} heterozygote hemizygous for *sev*^{dl2}. (M) Double heterozygote for *sE*²-*Dsrc41*^{KR} insertion and a *Dsrc41* deficiency. (N) Heterozygote for *sE*²-*Dsrc41*^{KRYF} insertion (KRYF-A).

morphological change in eye structure was detected in any of three transgenic lines with *sE*²-*Dsrc41*⁺ (Fig. 4C). This finding is important, as under similar conditions, flies with a single copy of the wild-type *Dsrc64* transgene showed extensively deformed eye morphology (Kussick et al. 1993).

Dsrc41⁺ and *Dsrc41*^{YF} transgenes were overexpressed in embryos by heat shock at 37°C for 30 min. Overexpressed *Dsrc41*⁺ gave no appreciable effect on viability, whereas the overexpression of *Dsrc41*^{YF} was deleterious.

Table 1. *sE-Dsrc41^{YF}* phenotype is affected by mutations in the *sev* signaling pathway

Genotype	Average number of R7-type neurons	Number of ommatidia
Wild type	1.0 ± 0.0 (±0.0)	234
WT-A	1.0 ± 0.0 (±0.1)	185
YF-A	2.7 ± 0.1 (±1.2)	362
<i>drk</i> ^{+/+} ; YF-A	1.0 ± 0.0 (±0.2)	250
<i>E (sev)1A^{ep}</i> ^{+/+} ; YF-A	1.0 ± 0.0 (±0.0)	200
<i>Sos^{x122}</i> ^{+/+} ; YF-A	1.1 ± 0.0 (±0.4)	398
YF-A/ <i>Ras1^{2F}</i>	1.1 ± 0.1 (±0.4)	387
<i>D-raf¹</i> ^{+/+} ; YF-A	1.3 ± 0.1 (±0.5)	267
<i>Dsor1¹</i> ^{+/+} ; YF-A	1.6 ± 0.1 (±0.8)	228
YF-A; <i>boss¹</i> ^{+/+}	1.9 ± 0.1 (±0.8)	340
YF-B	2.1 ± 0.1 (±0.9)	350
YF-B; <i>boss¹/boss¹</i>	0.6 ± 0.0 (±0.7)	367
<i>sev^{d2}</i> ^{+/+} ; YF-A	1.4 ± 0.1 (±0.6)	309
<i>flb^{3C81}</i> ^{+/+} ; YF-A	1.4 ± 0.2 (±0.6)	267
YF-A/ <i>E (sev)3B^{1E}</i>	2.4 ± 0.2 (±0.8)	284
YF-A/ <i>E (sev)3D^{0Q}</i>	2.6 ± 0.1 (±0.8)	200

For YF-A series, the transgene (*sE-Dsrc41^{YF}*) is heterozygous, whereas it is hemizygous in the case of YF-B. Two kinds of deviations are shown: The first one was calculated using the total number of eyes examined, and the second one, that of ommatidia. In the third column, only the total number of ommatidia examined is shown.

Close inspection of embryos homozygous for *Dsrc41^{YF}*, heat-shocked at 4–7 hr of development, and cultured at 25°C until 13–16 hr of development demonstrated that the overexpression of the activated *Dsrc41* results in the failure of CNS shortening and dorsal closure, leading to embryonic lethality (Fig. 3J,K). In contrast, the overexpression of wild-type *Dsrc64*, along with that of its activated version, has been shown to be embryonic lethal (Kussick et al. 1993). It may thus follow that like vertebrate c-Src, *Dsrc41* activity is repressed by stoichiometric phosphorylation of the carboxy-terminal conserved tyrosine residue (Tyr-511).

Wild-type *Dsrc41* is essential for normal eye morphogenesis

c-Src with an inactive ATP-binding site because of a Lys → Arg substitution has been shown to be capable of serving as an effective antagonist of wild-type c-Src and, hence, to exhibit Src kinase-negative phenotypes (Chang et al. 1995). Thus, by P-mediated transformation (Fig. 2D), four transgenic fly lines (KR-A, KR-B, KR-C, and KR-D) were made that expressed a dominant-negative version of *Dsrc41* (*Dsrc41^{KR}*) driven by the *sE²* enhancer.

All four transgenic fly lines exhibited similar rough eye phenotypes (Fig. 4D). Cobalt sulfide staining (Fig. 6C) showed the shape and distribution of preclusters to be considerably affected. Either R3 or R4 precursors, or both, appeared to stain more weakly than their wild-type counterparts. As shown below, rhodamine-phalloidin and anti-DE-cadherin staining suggested that putative

adherens junctions situated between R3 and R4 precursors is disorganized extensively in *Dsrc41^{KR}* flies.

H214 is an enhancer-trap marker used to identify precursors for extra R7-type neurons (Mlodzik et al. 1992). The ectopic expression of H214 first became discernible in a very small fraction of late *Dsrc41^{KR}* larvae (rows 17 and more posterior; Fig. 6H), and ommatidia having extra H214-positive cells increased in number extensively during pupal stages (Fig. 6L). This suggests that the formation of ectopic pro-R7s in *Dsrc41^{KR}* flies initiates at a stage much later than that for the formation of authentic R7 precursors.

During pupal stages, stacking of nuclei of two seven-up (*svp*)-positive cells became more apparent, and loss of *svp*-positive cells was observed in a small fraction of pupal ommatidia (Fig. 6J). In *Dsrc41^{KR}* adult eyes, rhabdomere patterns and ommatidial spacing were highly abnormal: Most ommatidia contained one or two additional R7-type neurons, whereas one or a few outer photoreceptors were absent from 50% ommatidia (Figs. 5I and 7). Taken together, these results indicate that the dominant-negative form of *Dsrc41* affects many steps of eye morphogenesis.

If, as expected, *Dsrc41^{KR}* serves as an antagonist of wild-type *Dsrc41*, eye phenotypes induced by the *Dsrc41^{KR}* transgene should be suppressed and enhanced, respectively, by the increment and decrement of the dose of wild-type *Dsrc41*. Figures 4, E and F, 5, J and M, 6, B and D, and 7 show this to be the case. All *Dsrc41^{KR}*-dependent phenotypes examined so far were suppressed and enhanced, respectively, by simultaneous expression of an additional copy of a transgene, *sE²-Dsrc41⁺* and one-copy reduction of the endogenous *Dsrc41* (Figs. 4E,F, 5J,M, and 6B,D). Note that the additional introduction of a heterozygous deficiency of the *Dsrc41* locus results in the absence of R3/R4 photoreceptors stained strongly by cobalt sulfide (Fig. 6D).

The *Dsrc41^{KR}* transgene might function only to titrate out a negative regulator (such as Csk) to increase the fraction of the active, unphosphorylated form of endogenous (wild-type) *Dsrc41*. Activated *Dsrc41* thus generated might undergo relocation to bind to target molecules (Kaplan et al. 1994) to consequently induce gain-of-function phenotypes similar to those observed in *Dsrc41^{YF}* flies. But this appeared unlikely, as (1) timing of the appearance of *Dsrc41^{KR}*-dependent defects differed considerably from that of *Dsrc41^{YF}*-dependent defects, and (2) *Dsrc41^{KR}* phenotypes were considerably enhanced by an additional YF mutation that may neutralize kinase activation by stimulating competitive inhibition upon target binding (Fig. 2D; see Fig. 5I,N). *Dsrc41* may thus be essential for normal eye morphogenesis.

boss-dependent, extra R7 formation in *Dsrc41^{YF}* ommatidia

R7 formation in the wild type depends on Sev kinase activated by Boss expressed on the surface of R8 (Kramer et al. 1991). Although R7 and cone cell precursors express Sev, only pro-R7s are capable of coming into con-

tact with R8 and receiving Boss signals (Tomlinson et al. 1987). This R7–R8 interaction may be demonstrated by genetic analysis along with Boss internalization (Reinke and Zipursky 1988; Kramer et al. 1991).

Table 1 and Figure 5, G and H, show that the number of extra R7-type neurons in *Dsrc41^{YF}* flies decreases considerably with the one-dose reduction of *boss* or *sev*. This is of interest, as no effect of *boss* on gain-of-function mutations of *Sev* pathway genes has been reported (Basler et al. 1991; Fortini et al. 1992). Also, as in the case of the authentic pro-R7 (Fig. 6E), Boss was found to be internalized in a small but appreciable fraction of *Dsrc41^{YF}* ommatidial cells (Fig. 6F), which are most likely to be precursors for extra R7-type neurons. Our preliminary experiment also indicated a one-dose reduction of *Dsrc41* to give no appreciable effect on *Sev^{S11}*

(data not shown), suggesting that *Dsrc41* functions upstream of *Sev*. *sE-Dsrc41^{YF}* would thus appear to endow cone cell precursors with the ability to make contact with R8 so as to receive the Boss signals for neuralization.

Requirement of the Ras1/MAPK signal transduction pathway beginning with Boss for both gain-of-function and dominant-negative phenotypes of Dsrc41

Genetic analysis using haploinsufficiency showed that the entire Ras1/mitogen-activated protein kinase (MAPK) signal transduction pathway is essential for *Dsrc41^{YF}*-dependent formation of R7-type neurons (Table 1); one-copy reduction of endogenous *drk*, *Sos*, or

Figure 6. (A–D) Cobalt sulfide staining of eye discs. (White triangles) Morphogenetic furrows. (A) Wild type; (B) double heterozygote for *sE²-Dsrc41^{KR}* and *sE²-Dsrc41⁺* insertions; (C) Heterozygote for *sE²-Dsrc41^{KR}* insertion (KR-A); (D) Double heterozygote for *sE²-Dsrc41^{KR}* insertion (KR-A) and a *Dsrc41* deficiency. (E) Boss internalization in wild-type pro-R7 (see arrowheads). (F) Boss internalization in homozygous eyes for *sE-Dsrc41^{YF}* insertion (YF-A). (Arrowheads) Boss incorporated into authentic and ectopic pro-R7s adjacent to the same R8. (G) *svp-lacZ* expression in larval eye discs heterozygous for *sE²-Dsrc41^{KR}* insertion. Arrowheads show irregularity in spacing between R3/4. (H) *H214* expression in a *sE²-Dsrc41^{KR}* transformant eye disc. (Arrowhead) Ectopic signals in cone cell precursors at the most mature stage. (I, J) *svp-lacZ* expression in pupal eye discs of wild-type (I) and in a *sE²-Dsrc41^{KR}* transformant line (J). (Arrows) Irregularity in spacing; (arrowhead) ommatidium containing only three *svp*-positive cells. (K, L) *H214* expression in pupal eye discs. (K) Normal expression in wild-type pro-R7. (L) Ectopic expression in *sE²-Dsrc41^{KR}* transformant line (see arrowheads). (M–W) Confocal images. (M, N, S, U) Anti-DE-cadherin antibody staining. (O, P) Anti-LacZ antibody staining. (Q, R) Double staining with anti-DE-cadherin (green) and anti-LacZ (red) antibodies. (T) Double staining with anti-DE-cadherin antibody (green) and rhodamine-phalloidin (dark blue). (V, W) Rhodamine-phalloidin staining. (M, N) DE-cadherin signals from the apical-most region and from the region 1–2 μ m beneath the

apical surface of the same row 8 sample, respectively. (I–III) Type I to type III signals. (O) Apical-most LacZ signals. (P) LacZ signals from the region 1–2 μ m beneath the apical surface. (O, P) Signals from the same sample in row 9. (Q, R) Row 9 and 10 signals at the 1–2 μ m level. Yellow indicates colocalization of cadherin and the *Dsrc41*–LacZ fusion protein. (S–W) Staining patterns near the morphogenetic furrow. (Arrowheads) Strong cadherin signals along row 5 R3–R4 boundaries. Light blue in T indicates colocalization of cadherin and actin fibers. The absence of cadherin-positive, putative adherens junctions (U) and actin cytoskeleton (W) along boundaries between row 5 R3/R4 expressing *Dsrc41^{KR}* is indicated by arrows.

Ras1 virtually completely suppresses ectopic neuralization. Enhanced effects of these elements may be partly attributable to the fact that not only the Boss/Sev signal but also the DER [*Drosophila* epidermal growth factor (EGF) receptor] signal contributes to neuralization in the *Dsrc41^{YF}* mutant (Table 1). However, unlike the Sev pathway in the authentic R7 (Simon et al. 1991), *cdc37* and *E[sev/3D]* may not be required for ectopic neuralization in the *Dsrc41^{YF}* mutant.

Dominant-negative phenotypes of *Dsrc41* also depend on the *Ras1*/MAPK signal transduction pathway beginning with Boss (Figs. 5 and 7). Lost outer photoreceptors were virtually completely recovered by one-copy reduction of either *Ras1* or *boss* or the absence of *sev*. The formation of extra R7-type neurons was also partially reversed (Figs. 5 and 7). However, it should be noted here that the Boss protein expressed in R8 may not be related to these phenotypes because neuralization of extra R7-like cells and loss of outer photoreceptors in *Dsrc41^{KR}* flies occur mainly during pupal stages when Boss expression is not specific to R8 but rather ubiquitous and non-functional (Kramer et al. 1991).

Localization of Dsrc41^{YF}-LacZ in the close vicinity of DE-cadherin-positive, putative adherens junctions

In vertebrate cultured cells, Src is relocalized to focal

adhesions following activation and controls cytoskeleton organization and cell signaling (Kaplan et al. 1994). Although no focal adhesions have been recognized in larval eyes, larval ommatidial cells are connected by adherens junctions (Longley and Ready 1995), which are presumably functional analogs of focal adhesions in the cell-cell adhesion system (Gumbiner 1996). In vertebrates, classic cadherins such as E-cadherin have been shown to be essential not only for formation of adherens junctions but also for the maintenance of cell-cell contacts and actin cytoskeleton (Takeichi 1991; Gumbiner 1996). Thus, we first examined the spatial distribution of DE-cadherin, a putative ortholog in *Drosophila* of vertebrate E-cadherin (Oda et al. 1994), and compared it with that of the *Dsrc41^{YF}*-LacZ fusion protein.

Figure 6M shows the distribution of DE-cadherin signals obtained by confocal microscopy, indicating that DE-cadherin distributes widely along boundaries of many ommatidial and nonommatidial cells. Because the distribution of actin fibers [cytoskeleton] visualized by rhodamine-phalloidin was very similar, if not identical, to that of DE-cadherin (Fig. 6T), DE-cadherin-positive regions found in larval eyes would appear to correspond to adherens junctions and/or their close relatives in developing eyes.

In normal eye development, ommatidial formation is

Figure 7. Suppression of *Dsrc41^{KR}* phenotypes. Numbers of R7-type and outer photoreceptors per ommatidium are indicated.

lineage independent, and each ommatidial precursor is assembled through the stepwise recruitment of cells into stereotyped clusters (for review, see Wolff and Ready 1993). Developing photoreceptors express neural antigen in a fixed sequence: R8 expresses neural antigens first and is followed by the R2/R5 pair and then by the R3/R4 pair. Interestingly, as shown in Figure 6S, the order and timing of the appearance of strong cadherin signals were virtually identical to those of neural antigens (for neural antigen expression, see Wolff and Ready 1993). Strong cadherin signals first appeared along boundaries of R8 in row 2. Photoreceptors R2 and R5 begin expressing strong cadherin signals in rows 3 and 4. Strong cadherin signals appeared along R3–R4 boundaries in row 5. It may thus follow that the formation of adherens junctions is initiated at a very early stage of photoreceptor development.

In row 9 or 10, three distinct types of DE-cadherin signals were recognized. Type I signals distributed along the apical-most boundaries of cells not yet recruited into ommatidia and putative cone cell precursors (Fig. 6M). Type II signals (0.5–2 μ m beneath the apical surface) were found along boundaries between cone cells and photoreceptors (Fig. 6N). Type III signals, $\sim$ 1 μ m in height and situated at $\sim$ 1.5 μ m beneath the apical surface, were readily apparent along boundaries between neighboring photoreceptor precursors (Fig. 6N). Locations of type II and type III signals appeared to coincide with those of zonula adherens visualized by electron microscopy (Longley and Ready 1995), suggesting that type II and type III signals may correspond to zonula adherens. Recently, this notion was confirmed further in an independent experiment (T. Uemura and H. Oda, pers. comm.).

The distribution of the fusion protein consisting of activated Dsrc41 and LacZ (*sE²-Dsrc41^{YF}LacZ*; Fig. 2D) was examined using anti-LacZ (or *Escherichia coli* β -galactosidase) antibody. *sE²-Dsrc41^{YF}-lacZ* flies exhibited eyes slightly rough in appearance. Confocal images of anti-LacZ and anti-DE-cadherin antibody double staining gave strong indication that Dsrc41–LacZ signals considerably overlap three types of DE-cadherin signals (Fig. 6R). However, it should be noted that type II signals of Dsrc41–LacZ exhibit marked apical extension (Fig. 6O), and the appearance of type III signals of DE-cadherin precedes those of Dsrc41–LacZ (Fig. 6, cf. Q and R). Taken together, these results suggest that activated Dsrc41 is localized in close proximity to DE-cadherin-positive adherens junctions and/or other types of DE-cadherin aggregates.

Failure in formation of stable putative adherens junctions between adjacent precluster cells expressing Dsrc41^{KR}

As shown in Figure 6C, a transient expression of *Dsrc41^{KR}* in precursors for R3 and R4 resulted in ommatidial disorders in preclusters. To examine whether these defects are related to possible alterations in cytoskeleton organization and/or cell–cell contacts, the distribution of DE-cadherin and actin fibers in *Dsrc41^{KR}* preclusters was examined. As shown in Figure 6, U and W, little or no signal for actin fibers and DE-cadherin could be de-

tected along boundaries between R3 and R4 precursors. DE-cadherin and actin fiber signals along R3/R2 and R4/R5 boundaries decreased considerably in intensity. Therefore, it may follow that the reduction of Dsrc41 kinase activity results in disorganization of cytoskeletal actin fibers and putative adherens junctions containing DE-cadherin, which is presumed to be responsible for cell–cell contacts (Gumbiner 1996).

Discussion

The protein encoded by *Dsrc41* shows hallmarks of a bona fide Src family member. Our results show that *Dsrc41* is a much closer relative of vertebrate c-src than is *Dsrc64*, long considered to be a unique gene belonging to the src family in *Drosophila* (Kussick et al. 1993). The phylogenetic tree in Figure 2C suggests that *Dsrc64* forms its own group distantly related to vertebrate src family members and *Dsrc41*. Kussick et al. (1993) showed dominant-negative and gain-of-function mutations of *Dsrc64* to cause severe eye defects. As in the case of *Dsrc41*, *Dsrc64* gain-of-function phenotypes are partially suppressed by reducing Ras-pathway signals, and thus *Dsrc64* may be considered to have functions similar to those of *Dsrc41*. Eye defects in *Dsrc41* gain-of-function mutants, however, are much less severe compared to those in *Dsrc64* mutants, and unlike wild-type *Dsrc64*, the overexpression of wild-type *Dsrc41* is not attended with any appreciable phenotype (Fig. 4C). These results and apparent differences in expression for *Dsrc64* and *Dsrc41* suggest that *Dsrc64* and *Dsrc41* are src relatives in *Drosophila*, each with different roles in cell differentiation and/or proliferation.

Is Dsrc41 required for control of cytoskeleton organization and cell–cell contacts?

In vertebrates, classic cadherins, including E-cadherin, are localized in zonula adherens or intercellular adherens junctions and are required to regulate cell–cell contact and tissue adhesion (Takeichi 1991). Carboxy-terminal portions of the cytoplasmic domain are essential for interaction with the cytoskeleton (actin fibers) and for cadherin-adhesive activity (Nagafuchi and Takeichi 1988). DE-cadherin shares in common many properties with vertebrate classic cadherins and is presumed to be a homolog of *Drosophila* (Oda et al. 1994). These investigators showed DE-cadherin to be present in zonula adherens in the case of embryos.

Our results showed DE-cadherin type II and type III signals to be located at $\sim$ 1 μ m beneath the apical surface of larval ommatidial cells, where zonula adherens junctions have been recognized by electron microscopy (Longley and Ready 1995). Thus, DE-cadherin-positive type II and type III signals are quite likely to correspond to adherens junctions in developing photoreceptor cells. Rhodamine–phalloidin staining showed that most signals for actin fibers colocalize with three types of DE-cadherin signals (Fig. 6T). Because classic cadherins constitute the actin-based zonula adherens (Takeichi 1991) in vertebrates, not only type II and type III, but also type

I, cadherin signals may be involved in regulation of cell-cell contacts and cytoskeleton organization.

By the immunostaining of activated Dsrc41 with a LacZ tag, the activated Dsrc41-LacZ fusion protein was shown predominantly to be located quite close to putative adherens junctions containing cadherin (Fig. 6Q). A transient expression of the dominant-negative form of Dsrc41 in preclusters was noted to cause failure at putative adherens junctions, not only in the formation or maintenance of the actin cytoskeleton but also in the deposit of DE-cadherin (Fig. 6U). Cobalt sulfide staining showed the differentiation of R3 and R4 to be affected by *Dsrc41^{KR}* expression (Fig. 6C). This effect was enhanced considerably by one-copy reduction of the endogenous wild-type *Dsrc41* gene (Fig. 6D). Thus, these results suggest that complexes consisting of Dsrc41, actin, and DE-cadherin and/or their localized concerted functions are essential for the formation and/or maintenance of cytoskeleton structures and cell-cell contacts, at least in precluster cells.

The disappearance of actin fibers and putative adherens junctions in precluster cells was observed only in dominant-negative mutants. Figure 6T shows the presence of actin fibers along with DE-cadherin in all precluster cells in wild-type and gain-of-function mutants. These findings cannot be explained by kinase-independent, amino-terminal activity of Src as proposed by Kaplan et al. (1994). The kinase activity of wild-type Dsrc41 may thus be considered essential for the formation and/or maintenance of the cytoskeleton and adherens junctions in precluster cells.

In more mature ommatidia, excess tyrosine kinase activity of Dsrc41 may affect cell-cell contact; as Figure 6F suggests, extra R7 cell formation in gain-of-function mutants of *Dsrc41* is partially dependent on ectopic contact between Boss-expressing R8 and cone cell precursors, which express Sev but cannot make contact with R8 during wild-type development.

Kaplan et al. (1994) showed the dephosphorylation of conserved carboxy-terminal tyrosine of c-Src to cause redistribution of c-Src to focal adhesions and to stimulate the Src-dependent phosphorylation of various cytoskeletal proteins including focal adhesion kinase (FAK). Fibronectin binding to integrins on NIH-3T3 fibroblasts promotes association among c-Src, Grb2 (Drk), and FAK, and the formation of an integrin-activated signaling complex linked to the Ras/MAPK signal transduction pathway (Schlaepfer et al. 1994). Analysis of mouse embryos lacking Csk demonstrated the importance of the dephosphorylation of Src family kinases in the regulation of embryonic cytoskeleton organization (Thomas et al. 1995). These findings and the present results for *Drosophila* suggest Src to be critically essential to the control of cell morphology and cell-cell contact in vertebrate and invertebrate development.

A part of the Ras1/MAPK signal may originate from Dsrc41 itself

Table 1 shows not only Sev but also DER to be involved in *Dsrc41^{YF}*-dependent neuralization. Vertebrate EGF re-

ceptor tyrosine kinase has been shown to be activated via association with v-Src (Maa et al. 1995). Therefore, similarly to the vertebrate EGF receptor, might be activated by *Dsrc41^{YF}*, the activated version of Dsrc41. In vertebrate cultured cells, v-Src, or the activated version of Src, is localized to focal adhesion contacts to phosphorylate FAK and/or p62, whose phosphorylation provides binding sites for Grb2 (Drk) and/or Sos (Richard et al. 1995). Our results indicate [activated] Dsrc41 to be localized in putative adherens junctions along with more apical regions. Thus, it is also possible that a part of the Ras signal for neuralization may originate from the activated Dsrc41 itself.

Materials and methods

Plasmids

pYC1.8, a P vector with a *vermillion* marker, was obtained from L. Searles (Fridell and Searles 1991). P plasmids expressing *Dsrc41* with or without mutations were made as follows. The *hsp70* promoter and polyadenylation signal, respectively, were introduced into the *Xba*I and *Hpa*I-*Not*I sites of pYC1.8M, a derivative of pYC1.8 with multicloning sites (F. Takahashi, unpubl.) to generate pVH9. Then, a 1.8-kb *Scal*-*Kpn*I fragment of *Dsrc41* cDNA with or without mutations (see below) was introduced into the *Bgl*II-*Hpa*I site of pVH9. Finally, one or two copies of a 670-bp *sev* enhancer (Bowtell et al. 1991) were introduced between the *Sal*I and *Sma*I sites just upstream of the *Dsrc41* minigene. *sE-Dsrc41^{YF}-lacZ* was made similarly. The *lacZ* fragment was introduced into the *Bam*HI site of Bluescript to generate pZSK-R. *Dsrc41^{YF}* was inserted into the *Kpn*I-*Pst*I site of pZSK-R. The resultant *Dsrc41^{YF}-lacZ* gene was introduced into pVH9, generating *sE²*.

Isolation of genomic DNA and cDNA clones

Genomic DNA and pupal cDNA clones of *Dsrc41* were isolated from libraries described previously (Kojima et al. 1991). For isolation of the first genomic DNA clone (*λgfk1*), *dtk5* (Shishido et al. 1991) was used as a probe. Hybridization was carried out at 55°C for 12 hr in H buffer [0.1 M Tris-HCl (pH 7.5) containing 1 M NaCl, 10 mM EDTA, 10× Denhardt's solution, 0.2% SDS, and 200 μg/ml of heat-denatured salmon sperm DNA], which includes ³²P-labeled probe DNA. Filters were washed at 55°C for 1–2 hr in 2× SSC [15 mM sodium citrate with 150 mM NaCl] containing 0.1% SDS. The second genomic DNA clone (*λgfk2*) was isolated using the cDNA insert of *λcfk* (see below) as a probe. cDNA clones were isolated using a 0.9-kb *Hind*III-*Kpn*I fragment of *λgfk1* (see line A in Fig. 1A) as a probe. Hybridization was performed at 65°C for 12 hr in H buffer, including ³²P-labeled, heat-denatured probe DNA. Filters were washed at 65°C for 1–2 hr in 0.1× SSC with 0.1% SDS.

Southern and Northern blotting

Canton-S DNA digested with suitable restriction enzymes was subjected to Southern analysis under less stringent conditions using a 0.9-kb *Hind*III-*Kpn*I fragment of *λgfk1* as a probe. Filters were hybridized at 60°C in H buffer for 24 hr and then washed at 60°C in 2× SSC with 0.1% SDS for 1–2 hr. Northern hybridization was carried out as described previously (Kojima et al. 1991).

In vitro mutagenesis

Tyrosine 511 of Dsrc41 was replaced by a phenylalanine residue using PCR, in which one of the primers contained a mutated

sequence. With the introduction of the YF mutant, the following primers were used: 5'-GGCGCTAGCAGCGCCAGATT-TCCC and 5'-AGTA-GGCCTGCGCCTCTTTGAAGTC (underline, mutated base). The KR mutant was generated by the Kunkel method (Sambrook et al. 1989) using 5'-TTCAGAGT-TCTAATTGCCAC as the antisense primer. All other procedures for molecular cloning were as described by Sambrook et al. (1989).

Germ-line transformation

A mixture of 300 µg/ml of *Dsrc41* plasmid DNA and 150 µg/ml of a helper plasmid, pUCHsΔ2-3, was injected into *v^{30f}; ry⁵⁰⁶* embryos (Spradling and Rubin 1982). Three [WT-A (first chromosome), B (third), and C (first)] and two [YF-A (third) and B (first)] viable transformant lines having wild-type and activated *Dsrc41* minigenes, respectively, were obtained. In the case of *Dsrc41^{KR}*, and *Dsrc41^{YF}-lacZ*, four and two transformant lines [KR-A (second), B (second), C (third), and D (third); DL-A (second) and B (second)] were obtained. In addition, two *Dsrc41^{KRYF}* lines [KRYF-A and KRYF-B] were recovered.

Fly stocks

Canton-S (wild type) and *v^{30f}; ry⁵⁰⁶* were our laboratory stocks, and *svp^{H162}*, an enhancer trap line of *svp* was obtained from G. Rubin (University of California, Berkeley). A fly strain having a *Dsrc41* deficiency [*In(2R)bw^{VD2L}Cy^R/Gla*] and *flb^{3C81}*, a DER mutant, respectively, were from Umca and Tübingen stock centers. The following were used as crosses to examine suppressor/enhancer activity: *y D-ral¹/Binsc, oc ptg* (Tsuda et al. 1993); *y w spl sn Dsor1¹/Binsc, oc ptg* (Tsuda et al. 1993); *Sos^{x122}/CyO* (Rogge et al. 1991); *y E(sev)1A^{OP}/Binsc, oc ptg* (Simon et al. 1991); *E(sev)2B^{OA} (drk)/CyO* (Simon et al. 1991); *E(sev)3B^{1F}/TM6B, Tb* (Simon et al. 1991); *Ras1^{e2F}/TM6B, Tb* (Simon et al. 1991); *E(sev)3D^{OC}/TM6B, Tb* (Simon et al. 1991); *sev^{d2}* (Simon et al. 1991); and *boss¹ h² ry⁵⁰⁶* (Kramer et al. 1991). Enhancer trap lines H214 and AE127 [*svp*] were obtained from Y. Hiromi (Princeton University, NJ).

Immunohistochemistry and in situ hybridization

Immunostaining and in situ hybridization were performed essentially according to Higashijima et al. (1992) and Shishido et al. (1993). Origins of anti-HRP, anti-LacZ (*E. coli* β-galactosidase) antibodies, and mAb22C10 were described in Higashijima et al. (1992). Anti-Boss and anti-DE-cadherin antibody (DCAD2) were gifts of L. Zipursky (University of California, Los Angeles) and T. Uemura (Kyoto University, Japan). A 1.9-kb *EcoRI*-*StuI* fragment of *Dsrc41* cDNA was used as a template for making RNA probe.

Electron microscopy

Samples for electron microscopy were prepared and observed as described previously (Kojima et al. 1991).

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