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The *Drosophila* Shark tyrosine kinase is required for embryonic dorsal closure

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Dorsal closure (DC) in the *Drosophila* embryo requires the coordinated interaction of two different functional domains of the epidermal cell layer—the leading edge (LE) and the lateral epidermis. In response to activation of a conserved c-Jun amino-terminal kinase (JNK) signaling module, the dorsal-most layer of cells, which constitute the LE of the stretching epithelial sheet, secrete Dpp, a member of the TGFβ superfamily. Dpp and other LE cell-derived signaling molecules stimulate the bilateral dorsal elongation of cells of the dorsolateral epidermis over the underlaying amnioserosa and the eventual fusion of their LEs along the dorsal midline. We have found that flies bearing a Shark tyrosine kinase gene mutation, shark1, exhibit a DC-defective phenotype. Dpp fails to be expressed in shark1 mutant LE cells. Consistent with these observations, epidermal-specific reconstitution of shark function or overexpression of an activated form of c-Jun in the shark1 mutant background, rescues the DC defect. Thus, Shark regulates the JNK signaling pathway leading to Dpp expression in LE cells. Furthermore, constitutive activation of the Dpp pathway throughout the epidermis fails to rescue the shark1 DC defect, suggesting that Shark may function in additional pathways in the LE and/or lateral epithelium.

[Key Words: Dorsal closure; Shark tyrosine kinase; epithelial sheets; *Drosophila* embryogenesis; Jun kinase]

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The synchronized movement of epithelial cell layers is crucial for the development of the body plan in metazoa. Folding, invagination, stretching and fusion of epithelia are accompanied by changes in cell shape, and the coordination of these changes involves interactions among the extracellular matrix, the cell surface, and the cytoskeleton (for review, see Leptin 1994). In the *Drosophila* embryo, gastrulation is followed by retraction of the germ band, which leaves the dorsal-most portion of the embryo covered by the extraembryonic membrane, termed the amnioserosa. The cells of the ectoderm, which give rise to the larval epidermis, cover the ventral and lateral portions of the embryo. In a process known as dorsal closure (DC), the cells from both sides of the ectoderm migrate over the underlying amnioserosa leading to the enclosure of the embryonic viscera. Movement of these epithelial layers depends exclusively on cell elongation and does not require mitotic growth or cell recruitment (for review, see Martinez-Arias 1993). DC has been divided into three phases (for review, see Knust 1997). Initially, the dorsal-most cells of the lateral epi-

dermis, which form the leading edge (LE) of the epithelial sheet, elongate along the dorsal–ventral axis and accumulate actin and nonmuscle myosin in their dorsal edges. Second, the more lateral epidermal cells lose their polygonal shape and also elongate, thereby moving the whole epidermis dorsally. Finally, the LE cells of both sides arrive at the dorsal midline and fuse. DC-defective mutations affect one or more steps of the ectodermal layer migration process, resulting in lack of cuticle secretion in the dorsal region, a characteristic dorsal open phenotype, and embryonic lethality (for review, see Martinez-Arias 1993).

The cellular events that characterize DC in *Drosophila*, along with an extensive collection of dorsal open mutations, make this an ideal system for analyzing the mechanisms orchestrating the movement of epithelial sheets. The recent molecular characterization of several of these loci has shed light on some of the signaling events involved in ectodermal cell stretching. Ectodermal expression of the TGFβ homolog, decapentaplegic (*dpp*), by cells of the LE is essential to promote migration of the lateral ectoderm (Riesgo-Escovar and Hafen 1997b). This domain of Dpp expression depends on activation of a Ser/Thr kinase cascade of members of the stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK) pathway (for review, see Kyriakis and...
Aavruch 1996]. Epistatic analysis and biochemical data indicate that missshapen [msn, a Ste-20-related kinase] functions upstream of hemipterous [hep, a MKK4-7 homolog] [Glise et al. 1995; Su et al. 1998]. hep, in turn, activates basket [bsk, a homolog of JNK] [Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996]. Phosphorylation of a fly Jun homolog [D-jun] by Bsk leads to the activation of an AP-1 type complex, presumably formed by D-Jun and D-Fos [kayak [kay]], which ultimately leads to transcriptional activation of Dpp only in cells of the LE [Riesgo-Escovar and Hafen 1997a; for review, see Noselli 1998, Noselli and Agnes 1999].

Movement of the epithelial sheet behind the LE depends on the receptor for Dpp encoded by the genes thick veins (tkv) and punt (put). In contrast to mutants of the JNK pathway, in which stretching of all cells of the ectoderm fails, cells of the LE undergo normal elongation in tkv and put mutants with no elongation of lateral cells [Riesgo-Escovar and Hafen 1997b]. All components of the Dpp signaling pathway are also important for DC as they also show a dorsal open phenotype on sensitized genetic backgrounds [Chen et al. 1998]. However, whether their role is to directly regulate lateral epidermal stretching or to promote the patterning of lateral cells, rendering them competent to receive additional cues that lead to their elongation toward the dorsal midline has yet to be determined [for review, see Leptin 1994].

The identification of several of the DC-defective loci has outlined a JNK–Dpp–Tkv/Put pathway to lateral ectoderm pathway. However, cloning and biochemical analysis of additional DC loci points to greater complexity in these signaling processes. For example, a JNK-independent pathway in the LE has been implicated in studies with Pkn, a Drosophila homolog of the putative Rho/Rac effector target family of serine/threonine kinases [Lu and Settleman 1999]. In addition, kay activity is not only required in the LE but also in the lateral ectoderm, and expression of Kay by cells of the lateral ectoderm depends on Tkv–Put activity [Riesgo-Escovar and Hafen 1997b]. It was also shown recently that the DC mutant myoblast city [mbc, Erickson et al. 1997] does not affect Dpp expression at the LE. Mbc is a homolog of the Rac-specific activator and integrin signaling protein DOCK180 [Kiyokawa et al. 1998; Nolan et al. 1998]. Because Mbc is expressed in the lateral ectoderm and not in the amnioserosa [Erickson et al. 1997], Mbc function is likely to be required only in the lateral ectoderm. These findings suggest either the existence of additional inputs that work in conjunction with Tkv–Put to contribute to the stretching of the lateral ectoderm in Drosophila embryos or novel mechanisms of signaling through Dpp receptors.

Recently we have described a Drosophila nonreceptor tyrosine kinase, termed Shark [SH2 domain ankyrin repeat kinase, Ferrante et al. 1995], which contains from amino to carboxyl terminus, a Src homology 2 (SH2) domain [N-SH2], five ankyrin repeats, a second SH2 domain [C-SH2], a proline-rich and basic region, and a tyrosine kinase domain. This study addresses the in vivo role of Shark in Drosophila development. Analysis of the phenotypes associated with a shark loss-of-function mutation demonstrate that Shark activity is essential for the migration of the dorsolateral epidermis of the embryo. We show that the Shark kinase functions in DC upstream of Dpp expression by LE cells.

**Results**

Isolation of a mutation in shark

*shark* was mapped previously by in situ hybridization to polytene segment 53A1 [Ferrante et al. 1995] on the right arm of the second chromosome. Further molecular and genetic mapping has localized the *shark* locus to the 52F interval [Fig. 1a]. The *shark* gene was found to be contained in a P1 clone [DS06638], cytologically mapped by the Berkeley Genome Project [www.fruitfly.org], to 52F2–52F8. In addition, independently mapped YAC clones were used to further confirm the chromosomal location. Clones DY506 [52F–53C] and DY664 [52E1–53A2], but not Dyr157–76 [52D7–52E9], were shown by PCR and Southern blot analysis to contain the entire *shark* gene. In situ hybridization to polytene chromosomes with the P1 clone DS06638 indicates that *shark* is removed by deficiencies *Df(2R)Jp7*, *Jp8*, and *Jp4* but not by *Jp1*, defining the position for the *shark* locus to a genetic interval between the proximal breakpoint of *Df(2R)*/*Jp8* and the distal breakpoint of *Df(2R)*/*P4* [Fig. 1a].

A search for recessive lethal mutations in the region uncovered by *Df(2R)*/*Jp8* identified 20 lethal complementation groups. Of these, 12 complementation groups were removed by *Df(2R)*/*Jp4* as well and represented candidate mutations in the *shark* locus. To determine if *shark* was represented by one of these lethal complementation groups and thereby encodes an essential function, we introduced transgenes expressing *shark* cDNA under the control of a heat shock promoter, into flies mutant for each complementation group in *trans* to the deficiency. The lethality of the single mutant allele of complementation group W-4 was the only phenotype rescued when *shark* expression was induced by heat shock treatment. This was true for either of the two *hs-shark* transgenic lines, and we therefore renamed the rescued mutation *shark* [Table 1]. Moreover, a *hs-shark* transgene encoding a catalytically inactive form [K698R] of Shark failed to rescue *shark* mutants, indicating that the Shark tyrosine kinase activity is required to rescue the mutant phenotype [data not shown].

Analysis of the lethal phase of the single *shark* mutant recovered indicated that either *shark* homoyzgoes, or *shark* / *Df(2R)*/*Jp7* or *shark* / *Df(2R)*/*Jp4* trans-heteryzygoes, resulted in pupal lethality [data not shown]. Sequencing of *shark* revealed the introduction of a stop codon at position 210 that predicts a protein containing only the first 210 amino acids of the *shark* ORF, which is likely to be nonfunctional, as two ankyrin repeats and the C-SH2, the proline-rich, and the tyrosine kinase domains are deleted completely. The truncation of the
shark ORF encoded by this mutant allele confirms the identity of the W-4 complementation group as the shark locus (Fig. 1b).

shark mRNA is expressed maternally

To localize the distribution of shark transcripts, we performed whole-mount in situ hybridization in wild-type embryos. Large amounts of shark mRNA were observed in ovarian nurse cells (not shown), unfertilized eggs, and syncitial blastoderm embryos (Fig. 2A). Owing to the abundant maternal contribution, shark message expression appears ubiquitous during early embryogenesis up until about stages 6–7 (stages according to Campos-Ortega and Hartenstein 1985). By the end of germ-band extension, shark mRNA is expressed primarily in the ectoderm. Following germ-band retraction, stages 12–15, the strongest shark mRNA expression occurs uniformly in the epidermis and other ectodermally derived structures such as the anterior foregut, hindgut, and Malphigian tubules.

Table 1. Heat shock-dependent rescue activity of the lethality of shark1/Df(2R)Jp4 flies by hs-shark-10 and hs-shark-2b transgenes

<table>
<thead>
<tr>
<th>Maternal genotype of Df(2R)Jp4 to test rescue activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percentage of shark1/Df(2R)Jp4 adults recovered at&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>22°C</td>
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<tr>
<td>w; hs-shark-2B, Df(2R)Jp4/CyO</td>
<td>20/350 (6%)&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>w, Df(2R)Jp4/CyO, hs-shark-10</td>
<td>30/400 (8%)&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup>Fly stocks were generated in which a balanced w; Df(2R)Jp4/CyO was placed in combination with two independent transgenic lines (hs-shark-2B and hs-shark-10, mapped to the X and third chromosomes, respectively) expressing a full-length shark cDNA under control of the heat shock promoter [hs-Casper, Thummel et al. 1988]. At least one allele of the 12 complementation groups mapped to the deficiency interval that contains shark (see text and Fig. 1a) was test-crossed to these hs-shark deficiency stock combinations to examine the ability of hs-shark-2B and −10 to rescue the lethality of any of the combinations l(2R)W1-12/Df(2R)Jp4.

<sup>b</sup>The number of CyO<sup>+</sup> shark1/Df(2R)Jp4 over the total number of flies, corresponding percentage in brackets.

<sup>c</sup>[HS] Heat shock treatment [1 hr at 37°C].

<sup>d</sup>Under conditions resulting in minimal rescue, shark1/Df(2R)Jp4 flies showed hep-like defects with variable penetrance ranging from 40% to 80% [see Fig. 3].
Expression patterns of *shark* mRNA [A–E] and protein [F–I,K–M] in wild-type embryos. [A–E] Whole mount in situ hybridizations with a full-length *shark* antisense [digoxin-labeled] riboprobe. Oriented, anterior to the left, arc syncytial [A, stages 2–3] and cellular [B, stages 4–5] blastoderm embryos showing large amounts of *shark* mRNA [lateral views]. (C) Lateral view of a germ-band-extended embryo [stage 9] showing ectodermal staining. (D) Dorsal view of stage 13 embryo. *shark* mRNA expression is seen in the epidermis, hindgut, and Malphigian tubules. By stages 15–16 (E, lateral view), *shark* mRNA levels have decreased significantly. [F–O] Shark protein expression in wild-type [F–I,K–M] and *shark*<sup>1</sup> GLC [J,N,O] embryos, detected with antisera raised against a GST fusion protein containing the *Shark* N-SH2 domain. Ubiquitous Shark protein in cellular blastoderm [F, stage 4] and at the onset of gastrulation [K]. Protein expression becomes more restricted during germ-band elongation [L, stages 8–10, lateral view]. Protein expression at stages 13–14 is primarily epidermal [G]. Shark protein expression at the LE (arrows) of the epidermis at stage 10 [M, lateral view, high magnification], stage 14 [H, dorsal view], and on completion of DC at stage 15 [I, dorsal view]. Staining is reduced in early [N], mid [J], and late [O] stage embryos derived from *shark*<sup>1</sup> GLCs [zygotic genotype *shark*<sup>1</sup>/*shark*<sup>1</sup>].

Rescue of a *shark* mutation suggests that *shark* functions in the JNK pathway

Wild-type *hs-shark* transgene rescue experiments without heat shock provided suboptimal rescue conditions (defined as those that yield progeny below the 33% of adults expected for a fully viable genotype, Table 1), which often gave rise to flies with a split thorax and/or *hep*-like defects [Fig. 3a]. This indicates that rescue of lethality can occur at levels of Shark that are insufficient to support normal thoracic closure. These phenotypes also point to a role for Shark in processes in which the Jun kinase kinase *hep* [Glise et al. 1995; Glise and Noselli 1997] and the *fos* homolog *kay* [Zeitlinger et al. 1997; Riesgo-Escovar and Hafen 1997a] have been shown to be involved. Both *hep* and *kay* have been shown to be components of the JNK signaling module, which regulates cell elongation in epithelial cell sheets undergoing morphogenetic stretching [Noselli 1998]. In addition to the split thorax defects, rescued *shark*<sup>1</sup>/[2R]p4; *hs-shark* females fertilized with sperm carrying the *shark*<sup>1</sup> allele gave rise to embryos that showed a DC-defective phenotype [Fig. 3b]. Like the split thorax phenotype, defects in embryonic DC have also been associated with loss of function in components of the JNK pathway that regulates *dpp* expression at the LE of the embryonic epi-
Figure 3. Phenotypes of the shark<sup>1</sup> mutation: (a) Adult phenotype of partially rescued flies. Dorsal view of the thorax of flies [anterior is left] of the following genotypes: (A) w; shark<sup>1</sup>/Ds(2R)Jp4; hs-shark-10/CyO; (B) hs-shark-10; (C) shark<sup>1</sup>/Ds(2R)Jp4; hs-shark-10 showing fully rescued flies, indistinguishable from wild type; (D) hs-shark-2B; shark<sup>1</sup>/Ds(2R)Jp4 flies showing mild defects due to incomplete rescue. A streak of naked cuticle is evident, marking the thoracic midline [arrow], (C,D) w; hs-shark-2B; shark<sup>1</sup>/Ds(2R)Jp4 under conditions of minimal rescue activity [no heat shock] showing a severe split thorax phenotype. The arrow points to a cleft running along the dorsal thoracic midline that results from incomplete movement toward the midline of both imaginal wing disc epithelia. The arrowhead in D points to the remnant of the left wing that failed to outgrow [hep-like defect; Riesgo-Escovar and Hafen 1997a; Zeitlinger et al. 1997]. (b) Lethality and dorsal open phenotype of shark<sup>1</sup> embryos derived from hs-shark-10 rescued shark<sup>1</sup>/Ds(2R)Jp4 females. (A) Cuticular pattern [lateral view] of a wild-type embryo, (B) mild cuticle defects [anterior open phenotype], (C) the more prevalent, strong dorsal open phenotype of embryos derived from rescued shark<sup>1</sup> females of the indicated genotype.

shark<sup>1</sup> homozygote germ-line clones exhibit a strong DC-defective phenotype

To investigate the phenotypic effects of the loss of Shark function, we eliminated maternal Shark activity by generating shark<sup>1</sup> homozygote GLCs [Chou and Perrimon 1996]. Zygotes derived from GLCs with the shark<sup>1</sup> mutation show a marked reduction in reactivity [Fig. 2N,J,O] in anti-Shark immunostaining. Because shark<sup>1</sup> is predicted to encode a truncated polypeptide containing the entire N-SH2 domain, which was the immunogen used to raise the antiserum in these experiments, the reduced immunoreactivity with the anti-GST/N-SH2 serum suggests that this allele produces a mutant protein that is expressed at low levels. This result is consistent with Western blot analysis of balanced shark<sup>1</sup> mutant embryos and adults, which also show low levels of the truncated protein predicted to be encoded by shark<sup>1</sup> [data not shown].

Analysis of cuticles derived from shark<sup>1</sup> homozygote GLCs fertilized with males of the genotypes shark<sup>1</sup>/CyO or Df(2R)Jp4/CyO [Fig. 4a] demonstrates that loss of maternal and zygotic Shark activity yields a strong DC-defective phenotype, similar to embryos produced by partially rescued shark<sup>1</sup>/Ds(2R)Jp4; hs-shark flies. Moreover, shark<sup>1</sup> GLCs fertilized with sperm carrying either the shark<sup>1</sup> allele or Df(2R)Jp7 give identical phenotypes, providing genetic evidence that shark<sup>1</sup> is a null mutation. Finally, zygotic rescue of all cuticular defects via the paternal allele, which leads to the eventual production of viable, fertile adults, indicates that Shark activity is not required for early embryonic events such as organization of the cellular blastoderm or gastrulation.

Shark participates in signaling in LE cells

Cuticles of maternal and zygotic shark null embryos are identical to those of strong alleles or maternal and zygotic loss-of-function alleles in hep, msn, bsk, kay, and D-jun mutants, among others. Cellular analysis of the epidermal cells in this group of mutants has revealed that the defect in cuticle secretion on the dorsal–anterior side, together with the failure to enclose the embryonic gut, is due to a defect in the directional elongation of the cells of the LE and lateral epidermis [Noselli 1998]. Immunostaining of epidermal cells with monoclonal antibodies to the band 4.1 homolog protein Coracle [Fehon et al. 1994] reveals that shark<sup>1</sup> has the same effect on epidermal cell function and epithelial cell morphogenesis [Fig. 4b, A–D] as all other members of the JNK pathway. Both LE and lateral epidermal cells fail to stretch toward the dorsal midline over the anterior three-quarters of the embryo, consistent with the deficient cuticle secretion in the anterior–dorsal region.

This defect, evident in all epidermal cells, demonstrates a requirement of Shark for the elongation of LE cells and suggests a role for Shark in the regulation of Dpp transcription, or the production of other signals activated in LE cells that subsequently target elongation of adjacent lateral cells. Because Shark is expressed throughout the epidermis, it is possible that it also has an active role in the lateral cells, operating downstream or in parallel to the Dpp/Tkv–Put pathway [Riesgo-Escovar and Hafen 1997b]. To investigate these possibilities, we analyzed Dpp expression by in situ hybridization in shark<sup>1</sup> null embryos and studied the effects of reconstitution of the JNK and the Dpp pathways, as well as Shark function, in a shark<sup>1</sup> null background by using a combination of upstream activating sequence (UAS) transgenes and Gal4 driver lines specific for the LE region and lateral cells of the embryonic epidermis.
Figure 4. Phenotypic analysis of shark1 embryos lacking maternal and zygotic Shark activity. (a) Cuticular phenotypes associated with complete loss of shark function (anterior to the left). [A,B] Lateral and ventral views, respectively, of representative cuticles derived from shark1 GLCs fertilized with shark1 chromosomes and showing a strong dorsal open phenotype. In this experiment females of the genotype FRT42B, ovo~D1/FRT42B, shark1 were crossed to shark1/CyO-balanced males, giving rise to two different zygotic genotypes: shark1[glo]/shark1 [maternally derived chromosome/paternally-derived chromosome], with the embryonic lethal phenotype shown in A and B and their fully paternally rescued siblings, shark1[glo]/CyO, shown in D. [C] shark1 GLCs fertilized with Df(2R)lp7 chromosomes show phenotypes indistinguishable from those in A and B, indicating that shark1 is a null mutation and that this phenotype is not due to any second site mutations in the JNK or the Dpp pathway could rescue the shark1 phenotype (Fig. 5C–J). When shark1 GLCs were crossed to Df(2R)lp7/CyO-balanced males, yielding two different zygotic genotypes: shark1[glo]/Df(2R)lp7, with phenotypes indistinguishable from those in A and B and the paternally rescued siblings, shark1[glo]/CyO. [D] The lethality of shark1 GLCs fertilized by a shark1-containing chromosome can be fully rescued by a wild-type paternal chromosome [a CyO balancer], which yields fertile adults. [b] Epidermal cell morphogenesis and dpp expression in wild-type [A,C] and shark1 mutant [B,D] embryos undergoing DC. The overall epidermal morphology is shown in whole-mount embryos [A,B], and the shape of epidermal cells, revealed by mAb anti-coracle antibody immunostaining in a wild-type embryo [lateral view, C] shows normal stretching in the direction of the dorsal midline. [D] Lateral view of a shark1 GLC that fails to complete DC showing that LE (arrows) and lateral epidermal cells maintain their polygonal shape and fail to elongate toward the dorsal midline. (c) Stage 14 embryos showing whole-mount in situ hybridizations to a dpp antisense riboprobe expression, in a paternally rescued shark1 GLC [A], which appears indistinguishable from wild type. (B) A shark1 GLC fertilized with a Df(2R)lp7 chromosome lacks expression of dpp message only at the LE. In both cases, the LE of the epidermis is indicated by an arrow.

A comparison of the embryonic dpp mRNA expression by whole-mount in situ hybridization, between wild-type and shark1 GLCs, showed that Shark is required for Dpp expression by cells of the LE following germ-band retraction. From stage 12 on, Dpp expression is absent in LE cells of shark1 GLCs [Fig. 4c], whereas expression in the ventral cord and midgut, which occurs in a JNK-independent manner [Noselli 1998], remains unaffected.

Consistent with the Shark expression pattern, the shark1 DC phenotype was completely rescued by expression of UAS–shark under control of the 69BGal4 driver, which expresses ubiquitously throughout the ectoderm [Fig. 5A,B]. Interestingly, however, expression of the LE region driver PnrGal4 in the same UAS–shark line failed to rescue the shark1 DC defect [data not shown]. This result, with a driver that expresses in three to five rows of the most dorsal ectodermal cells [Heitzler et al. 1996], suggests that either Shark expression is also required in the lateral epidermis or that Shark is required for Pnr-Gal4 expression in the LE region.

Because no obvious genetic interactions were obtained between Shark and mutations of the JNK pathway [data not shown], we tested whether constitutive activation of the JNK or the Dpp pathway could rescue the shark1 DC phenotype [Fig. 5C–J]. When shark1 GLCs were generated in the background of flies carrying a shark1 chromosome with an inserted transposon expressing an activated form of c-Jun [hs–SEjunAsp; Treier et al. 1995], shark1 DC defects were completely rescued, in some cases, as determined by the decreased penetrance of embryonic lethality (~10% lower than the fully penetrant 50% observed without the expression of hs–SEjunAsp) and by the complete or partial enclosure observed in unhatched embryos [Fig. 5C–F]. These results are consis-
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Figure 5. Effect of domain specific expression of Shark, activated c-Jun or activated Tkv on rescue of the dorsal open cuticle phenotype in shark¹ mutant embryos. [A,B] Embryos derived from shark¹ GLCs fertilized with sperm carrying the shark¹ allele, expressing UAS–shark¹ and 69B–Gal4 show complete rescue of the dorsal open phenotype. Furthermore, these embryos develop normally to fertile adults (data not shown). [C–F] Embryos derived from shark¹ GLCs fertilized with sperm carrying the shark¹ allele, and expressing an activated form of c-Jun under control of the heat shock promoter (hs-SEjunAsp). Partial rescue [C,D] or complete rescue [E,F] are readily observed and account for ~35% of the shark null embryos (the percentage of severe DC-defective embryos is reduced to ~15%), when embryos are heat-shocked between 5 and 10 hr after egg laying. [G,H] Embryos from shark¹ GLCs fertilized with a CyO balancer (paternally rescued) and carrying UAS–Tkv* and 69B–Gal4 show partially dorsalized cuticles that are indistinguishable from those seen in wild-type embryos expressing UAS–Tkv* and 69B–Gal4. [I,J] Embryos derived from shark¹ GLC fertilized with sperm carrying the shark¹ allele, expressing UAS–Tkv* and 69B–Gal4 show partial dorsalization (lack of ventral denticle belts) and no rescue of the dorsal open phenotype. The genotypes are written with the maternally derived chromosome on top [e.g., shark¹(gle)] and the paternally derived chromosome below [e.g., shark¹, hs-SEjunAsp]. [A,C,E] Ventral views; [G,I] dorsal views; [B,D,F,H,J] lateral views.

tent with the action of Shark upstream of bsk [JNK] in the JNK pathway in LE cells.

If Shark was required solely for Dpp expression in LE cells, expression of a constitutively activated Tkv receptor [Tkv*] under the control of the 69B driver throughout the ectoderm should rescue the shark¹ DC phenotype. Although embryos expressing the combinations of UAS–Tkv* and 69B–Gal4 can be readily identified by the partial dorsalization of their cuticles [Fig. 5G–J], two distinctive kinds of partially dorsalized cuticles were observed: (1) those that should be expressing the combination of transgenes in a paternally rescued background and that showed complete enclosure (~12%), and (2) an equivalent percentage of embryos, showing marginal or no rescue of the DC defect, in spite of being dorsalized, demonstrating expression of both Tkv* and 69B–Gal4. Because msn and bsk mutations are rescued by 69B–Gal4-driven UAS–Tkv* [Riesgo-Escovar and Hafen 1997b; Su et al. 1998], these findings raise the possibility that Shark may also function in an additional signal generating pathway in LE cells, in the lateral epidermis, or both.

Discussion

We have described a mutation in the nonreceptor tyrosine kinase Shark and the characterization of the phenotypes observed in ectodermal epithelial layers caused by loss of Shark function. As shown by the Shark requirement for Dpp expression and the rescue with activated Jun, our results indicate that it regulates morphogenesis of the embryonic epidermis. Shark is essential for activation of the JNK pathway in LE cells.

shark loss of function results in phenotypes similar to a subset of defects associated with loss of Dpp signaling activity in migrating ectodermal epithelial layers

Temperature-dependent rescue of the lethality of shark mutants with heat shock-driven cDNA transgenes resulted in dramatic adult phenotypes that suggested a role for Shark in morphogenesis of ectodermal epithelia. Adult flies expressing insufficient levels of Shark exhibit a split thorax phenotype, indicating a role for Shark activity in the dorsal joining of the wing imaginal epithelia derived from the left and right wing discs during pupal development. Unilateral deletions of wings and a cleft along the middle of the dorsal thorax have been reported in homozygous escapers of weak hep alleles [Glise et al. 1995] and partially rescued kay mutants [Riesgo-Escovar and Hafen 1997a; Zeitlinger et al. 1997]. As both hep and kay are components of the JNK/Dpp/Tkv–Put pathway, shown to be essential for epidermal cell sheet elongation and fusion during embryonic DC [Noselli 1998], it was proposed that wing disc epithelial stretching may be regulated by a JNK/Dpp/Tkv–Put pathway similar to the one operating in DC [Riesgo-Escovar and Hafen 1997a]. Recently, Zeitlinger and Bohmann (1999) have formally demonstrated that thorax closure is controlled by the same molecular events that regulate DC, in which the JNK pathway promotes LE expression of Dpp and Puck-
erated. Consistent with this, mutations that affect Dpp signaling show a split thorax and/or hep-type defect[s] under a variety of hypomorphic conditions that yield adult flies. For example, this is the case in viable tkv (Terracol and Lengyel 1994), mad (Raftery et al. 1995), and med (Hudson et al. 1998) heteroallelic combinations, as well as in tkv6/tkv6 adults, whose phenotypes have been enhanced by loss of function in one copy of the TGFβ-like 60A locus [also known as glass bottom boat (gbb)], mad, med, or put [Chen et al. 1998].

Adult shark² rescued females also showed reduced Shark accumulation in developing eggs as evidenced by the production of shark² homozygous embryos with strong DC zygotic defects. Considering that homozygous shark¹ mutants die as pupae and the early preblastoderm expression of shark mRNA is high, it seems plausible that the maternally derived Shark activity is sufficient to facilitate embryonic development. The requirement of Shark for DC was ultimately confirmed by removing Shark activity through the generation of shark mutant GLCs. Even though the shark mutant phenotypes that we have described are consistent with loss of Dpp expression and/or failure of signaling by Dpp receptors, unlike some other mutations in these pathways—dpp, tkv, put, mad, and med—which affect pattern formation and cell determination during eye, wing, and leg imaginal disc development (Raftery and Sutherland 1999), the shark² mutation does not seem to affect the development of imaginal disc-derived structures, aside from dorsal fusion of the thorax. That is, typical rough eye phenotypes, abnormalities in wing vein patterning, lack of distal tarsal segments in the adult legs, as well as dorsoventral patterning defects in the embryo, were not observed in shark¹ mutants. Thus, as with other loci controlling the epidermal LE-specific JNK pathway (Raftery and Sutherland 1999), the shark² mutation may not cause the development of imaginal disc-derived structures. The role of dorsal fusion of the thorax. That is, typical rough eye phenotypes, abnormalities in wing vein patterning, lack of distal tarsal segments in the adult legs, as well as dorsoventral patterning defects in the embryo, were not observed in shark¹ mutants. The role of Shark in the development of imaginal disc-derived structures is the subject of future study.

Shark activity is required in LE cells of ectodermal cell layers undergoing Dpp-dependent migration and stretching

To date, two functionally different cell types have been defined among the ectodermal cells of the embryo that eventually differentiate into cuticle-secreting epidermis: (1) LE cells, and (2) the cells of the lateral epidermis. In response to extracellular cues believed to emanate from the underlying amnioserosa, ectodermal LE cells are specifically induced to secrete Dpp, from mid- to late-embryogenesis. LE expression of Dpp is not only essential to coordinate developmental processes within the ectoderm itself but also to promote patterning of the juxta-posing dorsal mesoderm (Xu et al. 1998). In addition to the ability to transduce a signaling cascade mediated by a SAPK/JNK cascade that leads to Dpp expression, the morphology of the LE cells is dramatically altered following germ-band retraction, when the LE cells elongate along the dorso–ventral axis as they migrate in the direction of the dorsal midline (Noselli 1998). Dpp secretion by LE cells triggers a response in the immediately adjacent cells of the epidermis, which results in the concerted elongation of the entire epidermis along the dorso–ventral axis and enclosure of the dorsal side of the embryo. This response in lateral epidermal cells is transduced by the type I and II heterodimeric Dpp receptors Tkv and Put. Analysis of the effect of the combined loss of maternal and zygotic shark function, with markers that reveal changes in epidermal cell shape and function in the developing embryo, indicate that elongation of all cells of the epidermis is equally affected. This results in a strong DC-defective cuticular phenotype that is caused, at least in part, by loss of Dpp expression by LE cells, as revealed by Dpp in situ hybridization.

However, apart from its involvement upstream of the JNK pathway in LE cells, our transgenic rescue experiments suggest that Shark is either required in another pathway in the LE, in a pathway other than the Dpp pathway in the lateral epidermis, or both. Shark is expressed throughout the ectoderm, and expression of Shark driven by the 69B driver in both the LE and the lateral epidermis completely rescues the shark¹ DC phenotype. Consistent with Shark regulation of JNK or JNK-like pathways, the shark¹ DC phenotype is rescued by ubiquitous expression of activated c-Jun. The failure of 69B-Gal4-driven activated Tkv receptor to rescue the shark¹ DC phenotype is consistent with the possible involvement of Shark in novel pathways in the lateral epidermis, or for the generation of LE signals in addition to Dpp, for which evidence has been obtained recently [Lu and Settleman 1999]. Additional investigation of these possibilities is warranted.

The current findings clearly indicate that Shark regulates Dpp expression in LE cells [Fig. 6]. The discovery that Shark is a member of this pathway should assist in the identification of additional upstream components. Recent studies indicate that a Drosophila e-srC homolog, Src42A, also functions upstream of the JNK module in DC (Tateno et al. 2000). Consistent with both observations, during DC, tyrosine phosphorylation is prominent at the dorsal border of LE cells and is also found along the cell peripheries in the lateral epidermis (Harden et al. 1996). We speculate that Shark is an upstream regulator of multiple Ser/Thr protein kinase cascades, which regulate different biological effects, and that Shark transduces a signal(s) that is diversified in a manner analogous to receptor tyrosine kinase-mediated signaling that promotes pleiotropic effects in a variety of cell types.

Materials and methods

Genetic protocols

To screen for shark mutants, an EMS mutagenic screen for point mutations that fail to complement the deficiencies Df(2R)Jp8 and Df(2R)Jp4 was performed, essentially as described by Lewis and Bacher (1968). A detailed description will be published elsewhere (Z. Lin and R. Steward, unpubl.). The cytological characterization of the deficiency chromosomes was...
Rescue activity was assessed by scoring for survival of gion to test for the ability of the transgene to complement. Flies of the genotypes w; hs-shark-2B; Df(2R)Jp4/ciency stock. Flies of the genotypes were mated to a hs-shark-10

Figure 6. Schematic representation of Shark function in DC. The scheme summarizes our results, which indicate that Shark functions upstream of the JNK pathway in the LE, to regulate Dpp expression. The scheme also includes a JNK-independent pathway required in the LE that was proposed by Lu and Settlement (1999). Such a pathway might be a target for additional signals generated by Shark. In addition, Shark might also function in the lateral epidermis to regulate stretching of lateral epithelial cells [see text].

confirmed by testing their lethality in trans to single P-element lethal insertions mapped by the Berkeley Genome Project. To attempt P-element-mediated rescue of potential shark mutations, a full-length cDNA devoid of 5' and 3' UTRs was subcloned into the BglII site of the transformation vector pCasperhs [Ashburner 1989]. Two independent insertion lines, one on the X-chromosome [hs-shark-2B] and one on the third chromosome [hs-shark-10] were mated to a w; Df(2R)Jp4/Cyo deficiency stock. Flies of the genotypes w; hs-shark-2B; Df(2R)Jp4/ Cyo or w; Df(2R)Jp4/Cyo; hs-shark-10 were crossed to alleles of each of the lethal complementation groups in the shark region to test for the ability of the transgene to complement. Rescue activity was assessed by scoring for survival of CyO+ flies from crosses that were performed at 25°C, as well as crosses that were heat-shocked once a day for 1 hr at 37°C, from the first larval instar to late third larval instar stage of development.

To remove both maternal and zygotic Shark activity, GLC combinations and generation of GLCs was carried out by crossing hs-FLP7; FRT42B, shark 1/Cyo males [Chou et al. 1993]. To ensure survival of Canton S females at 25°C, as well as cross to w; shark1/Cyo males [Chou et al. 1993].

The heat shock and UAS-driven Shark expression plasmids used in rescue experiments were generated as follows: The shark ORF [only coding region] devoid of all UTR sequences was amplified by PCR using a shark cDNA [Ferrante et al. 1995] with the primers 5'-CCCAAAAGGATCCGGAGTGTGAT- CCCATG-3' and 5'-CATATAGCTGAGAAATTGCAC- TCAGC-3'. PCR reactions were carried out with Pfu polymerase [STRATAGENE], digested with NcoI and NotI, and subcloned into the plasmid pBP4 (Driever et al. 1990). The insert was digested with BglII and subcloned into the expression vectors pCasper-Shark and pUAST-Shark. Functionalized of these constructs and the loss of catalytic activity of the K69R mutated shark constructs were confirmed by transient transfection into Schneider cells, followed by anti-Shark and anti-phosphotyrosine Western blotting. P-element-mediated transformation of hs-Casper-Shark and pUAST-Shark was done according to Spradling and Rubin (1982).

Antibodies, immunostaining procedures, cuticle preparations, and photography

The antipeptide antibodies to the kinase domain of Shark have been described previously [Ferrante et al. 1995]. Polyclonal antiseras against a GST fusion protein (pGex-KG; Guan and Dixon 1991) containing the N-SH2 domain of the shark ORF [residues 9–119, Ferrante et al. 1995] were raised in rabbits (Cocalico Biologicals, Inc.) by injecting glutathione–agarose affinity-purified GST fusion protein [Smith and Johnson 1988]. Antibodies to β-galactosidase, used to identify embryos that did not carry a balanced chromosome containing a lacZ transgene (Cyo, eve- lacZ, or TM6 Tb, ubx-lacZ) were purchased from Sigma [mouse polyclonal, diluted 1:1000] and Cappel [rabbit polyclonal, diluted 1:3000]. Anti-Coracle mAbs C566.9 and C615.16 were generously provided by Richard Fehon [Duke University, Durham, NC], and were used together at a 1:400 dilution. Anti-Shark N-SH2 antiserum [used at a 1:4000 dilution], as well as all polyclonal antibodes, were preadsorbed against wild-type embryos prior to use.

Whole-mount immunostaining of embryos was carried out as described [Ashburner 1989]. Primary antibodies were detected with the ABC-elite system [Vector Laboratories]. Immunostained embryos were mounted in 90% glycerol and photographed under DIC optics [Zeiss Axiovert] using Ektachrome 160T film.

Cuticles were prepared essentially as described [Wieschaus and Nüsslein-Volhard 1986] and photographed under phase-contrast microscopy using Ektachrome 160T film. Photographic slides were scanned with a Polaroid Sprintscan 35 scanner and processed with Adobe Photosop 4.0 for Macintosh. Composite figures were prepared with Adobe Illustrator 6.0.

In situ hybridizations

Third instar larva polytenic chromosome squashes were prepared according to Pardue (1986) and probed by in situ hybridization with a biotin-labeled DNA fragments [Engels et al. 1986]. Whole-mount in situ hybridization of embryos was carried out according to Tautz and Pfeifle (1989) using digoxigenin-labeled RNA probes prepared by in vitro transcription [Boehringer Mannheim].

Molecular characterization of the shark1 allele

shark1 mutant flies were rescued in trans to Df(2R)Jp7 by the hs-shark-10 transgene. Genomic DNA was prepared from flies of the genotype w; shark1/Df(2R)Jp7; hs-shark-10 and used as template for subsequent PCR procedure. Primers used to amplify from the mutant genome are primer 1, 5'-CAAA-A CGCAACCAGGATGGAGTCATGCGCCG-3', primer 2, 5'-GAATTGGTAACAAGGTGCGACGCGAAGTGTAAGCG-3', primer 3, 5'-CG-
GTGCGACGTCAATCTCAATGG-3′; and primer 4, 5′-GC-GGATCCGATCTGGGTCCAGATCGTTAATACG-3′. Primers 1 and 3 were used to amplify the 5′ half of the shark gene; primers 2 and 4 for the 3′ half. Primers 1 and 4 are located in the 5′ and 3′ UTR [shark cDNA used in the rescue construct lacks all UTR sequences], permitting selective amplification of the mutant genomic sequence.

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References


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Erratum

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The Drosophila Shark tyrosine kinase is required for embryonic dorsal closure
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