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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.

The synchronized movement of epithelial cell layers is crucial for the development of the body plan in metazoans. 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

Avruch 1996). Epistatic analysis and biochemical data indicate that *misshapen* (*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, LE 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 DOCK-180 (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 (<u>SH</u>2 domain <u>a</u>nkyrin <u>r</u>epeat <u>k</u>inase, 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 DYR15-76 (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 *DfJp(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 *shark¹* (Table 1). Moreover, a *hs-shark* transgene encoding a catalytically inactive form (K698R) of Shark failed to rescue *shark¹* 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 *shark¹* homozygotes, or *shark¹/Df(2R)Jp7* or *shark¹/Df(2R)Jp4 trans*-heterozygotes, resulted in pupal lethality (data not shown). Sequencing of *shark¹* 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

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Figure 1. Schematic representation of the *shark* locus mapping and basis of the *shark[1]* mutation. (*a*) *shark* locus mapping. A chromosomal in situ hybridization, in which a P1 clone (DS06638) containing the *shark* gene was used to probe a pair of homologs of the genotype *Df(2R)Jp7/+,* is shown at the *top,* aligned with a drawing of the banding pattern of this region (Lindsley and Zimm 1992). The hybridization signal appears only on the wild-type chromosome. Molecular mapping of the *shark* gene to *Drosophila* genomic P1 (DS06638) and YAC (DY506, DY664, DYR15-76) clones was done by PCR analysis to test for *shark* coding sequences, and Southern blot analysis to establish presence of the entire *shark* gene. The cytological boundaries of the deficiency chromosomes *Df(2R)Jp1, Df(2R)Jp4, Df(2R)Jp7,* and *Df(2R)Jp8,* which we confirmed or rectified by viability over cytologically single P-element lethal insertions from the Berkeley Genome Project, are shown below the genomic clones. The shaded region highlights the *shark* mutant group (W-4), lined up to the deficiency chromosomes and genomic clones. (*b*) Representation of the wild-type and *shark[1]* ORFs. The *shark[1]* locus encodes a truncated form of Shark protein due to a C → T transition at nucleotide 685 that changes Gln-210 to a stop codon.
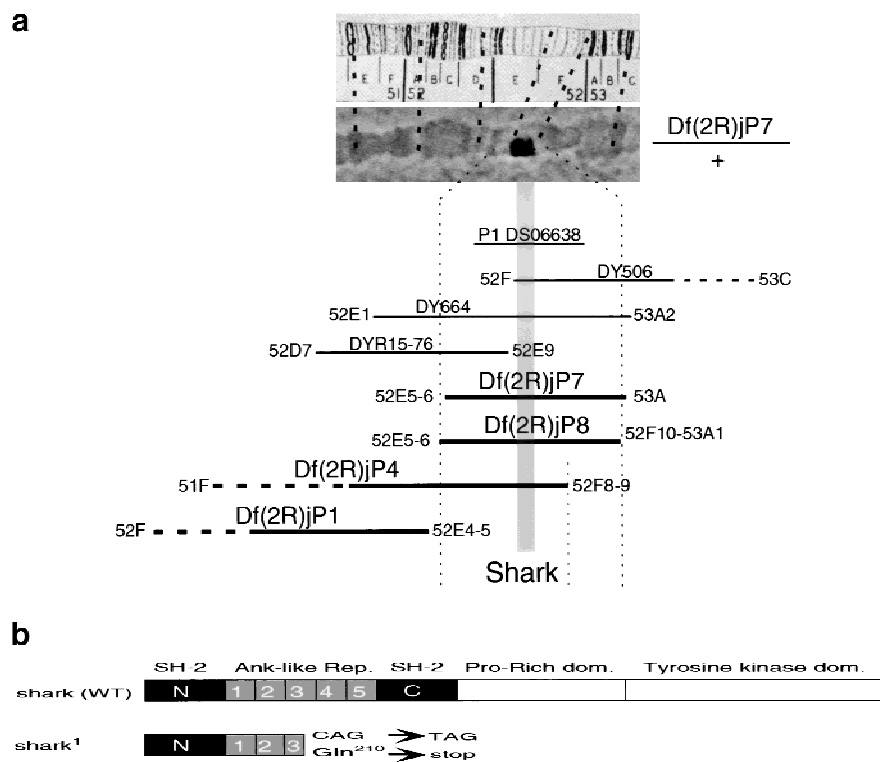
shark ORF encoded by this mutant allele confirms the identity of the W-4 complementation group as the *shark* locus (Fig. 1b).

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 Malphi-

Table 1. *Heat shock-dependent rescue activity of the lethality of* shark[1]/Df(2R)Jp4 *flies by* hs-shark-10 *and* hs-shark-2b *transgenes*
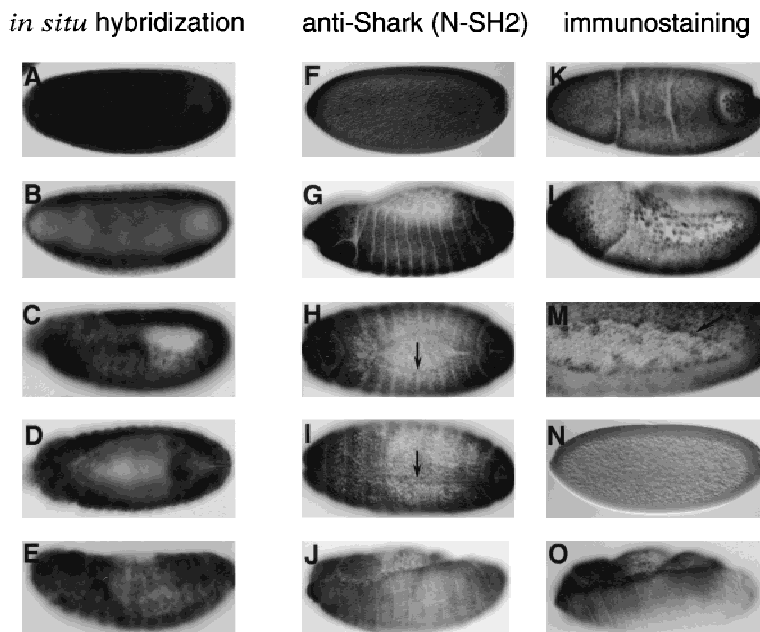
Maternal genotype of *Df(2R)Jp4* to test rescue activity[a]	Percentage of *shark[1]/Df(2R)Jp4* adults recovered at[b]		
	22°C	27°C	27°C (+7 HS)[c]
w; hs-shark-2B; Df(2R)Jp4/CyO	20/350 (6%)[d]	45/336 (13%)	80/234 (34%)
w; Df(2R)Jp4/CyO; hs-shark-10	30/400 (8%)	50/340 (15%)	81/230 (35%)

[a]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.*
[b]The number of *CyO[+]* [*shark[1]/Df(2R)Jp4*] over the total number of flies; corresponding percentage in brackets.
[c](HS) Heat shock treatment (1 hr at 37°C).
[d]Under conditions resulting in minimal rescue, *shark[1]/Df(2R)Jp4* flies showed *hep*-like defects with variable penetrance ranging from 40% to 80% (see Fig. 3).

in situ hybridization   anti-Shark (N-SH2)   immunostaining

Figure 2. 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*, are syncitial (*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[1]* GLC (*J,N,O*) embryos, detected with antiserum 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[1]* GLCs (zygotic genotype *shark[1]/shark[1]*).

gian tubules. Most of the *shark* message is down-regulated by the end of embryogenesis (Fig. 2A–E). Protein expression was analyzed using an antiserum raised to the Shark N-SH2 domain. In general, staining with this antiserum paralleled the expression of *shark* mRNA (Fig. 2F–I, K–M). Shark protein is expressed abundantly from the onset of cellularization, resulting in ubiquitous expression until the commencement of gastrulation. From this stage on, its expression is most prominent in the ectoderm and some of the ectodermally derived tissues. At stage 10 (full germ-band extension) and thereafter, Shark protein expression increases again but is restricted to the ectoderm, foregut, hindgut, and Malphigian tubules. During germ-band extension (stages 9–10) and in migrating epidermal cells during DC (stage 13–14), Shark protein expression is particularly strong at the LE of the epidermis. By the end of embryogenesis, epidermal expression decreases. Embryos homozygous for deficiency *Df(2R)Jp7*, which removes the *shark* locus, as well as *shark[1]* homozygote embryos, show a reduction in immunoreactivity only after stages 13–14 (data not shown), consistent with a substantial contribution of maternal mRNA. In contrast, staining is reduced in embryos derived from *shark[1]* germ-line clones (GLCs; see below) (Fig. 2N,J,O), consistent with the specificity of the antiserum for Shark, which could detect residual mutant protein. Also, in agreement with genetic evidence for a predominant role of Shark in the epidermis (see below), late *shark[1]* GLC-derived embryos consistently lack all immunoreactivity in epidermal cells (Fig. 2O). This pattern of Shark protein expression differed significantly from the previously reported immunolocalization of Shark protein with antibodies directed to the kinase domain (Ferrante et al. 1995), where poor pregastrulation

and epidermal staining was seen that was contributed to by the dominance of apical staining of epithelia and staining of the tracheal system by one of three antibodies in the mixture of antipeptide antibodies used. This antibody was subsequently shown to detect an unrelated epitope (R. Fernandez, unpubl.).

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[1]/Df(2R)Jp4; hs-shark* females fertilized with sperm carrying the *shark[1]* 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 *shark¹* mutation: (*a*) Adult phenotype of partially rescued flies. Dorsal view of the thorax of flies (anterior is *left*) of the following genotypes: (*A*) *w; shark¹/Df(2R)Jp4; hs-shark-10/hs-shark-10* showing fully rescued flies, indistinguishable from wild type; (*B*) *w; hs-shark-2B; shark¹/Df(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; shark¹/Df(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 *shark¹* embryos derived from *hs-shark-10* rescued *shark¹/Df(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 *shark¹* females of the indicated genotype.
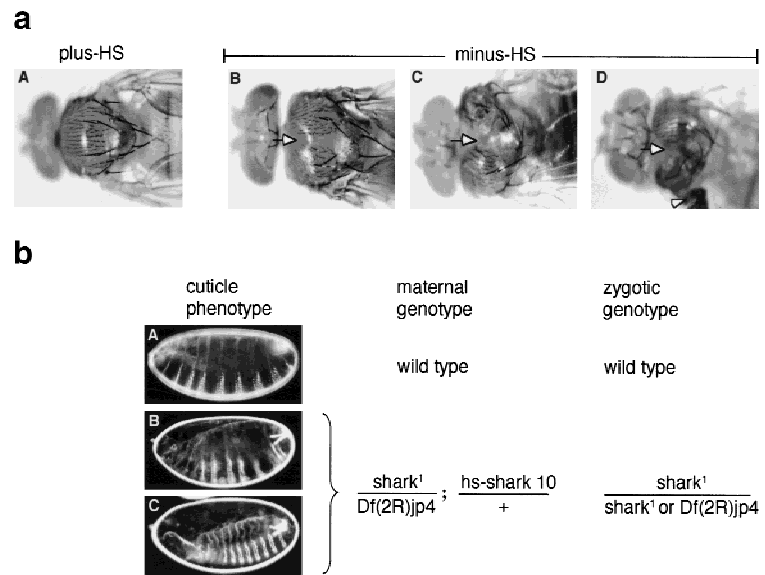
dermis (Riesgo-Escovar and Hafen 1997b; Zeitlinger et al. 1997; Zeitlinger and Bohmann 1999).

### shark¹ *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 *shark¹* homozygote GLCs (Chou and Perrimon 1996). Zygotes derived from GLCs with the *shark¹* mutation show a marked reduction in reactivity (Fig. 2N,J,O) in anti-Shark immunostaining. Because *shark¹* 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 *shark¹* mutant embryos and adults, which also show low levels of the truncated protein predicted to be encoded by *shark¹* (data not shown).

Analysis of cuticles derived from *shark¹* homozygote GLCs fertilized with males of the genotypes *shark¹/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 *shark¹/Df(2R)Jp4; hs-shark* flies. Moreover, *shark¹* GLCs fertilized with sperm carrying either the *shark¹* allele or *Df(2R)Jp7* give identical phenotypes, providing genetic evidence that *shark¹* 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 *shark¹* 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 *shark¹* null embryos and studied the effects of reconstitution of the JNK and the Dpp pathways, as well as Shark function, in a *shark¹* 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.

a

b

c
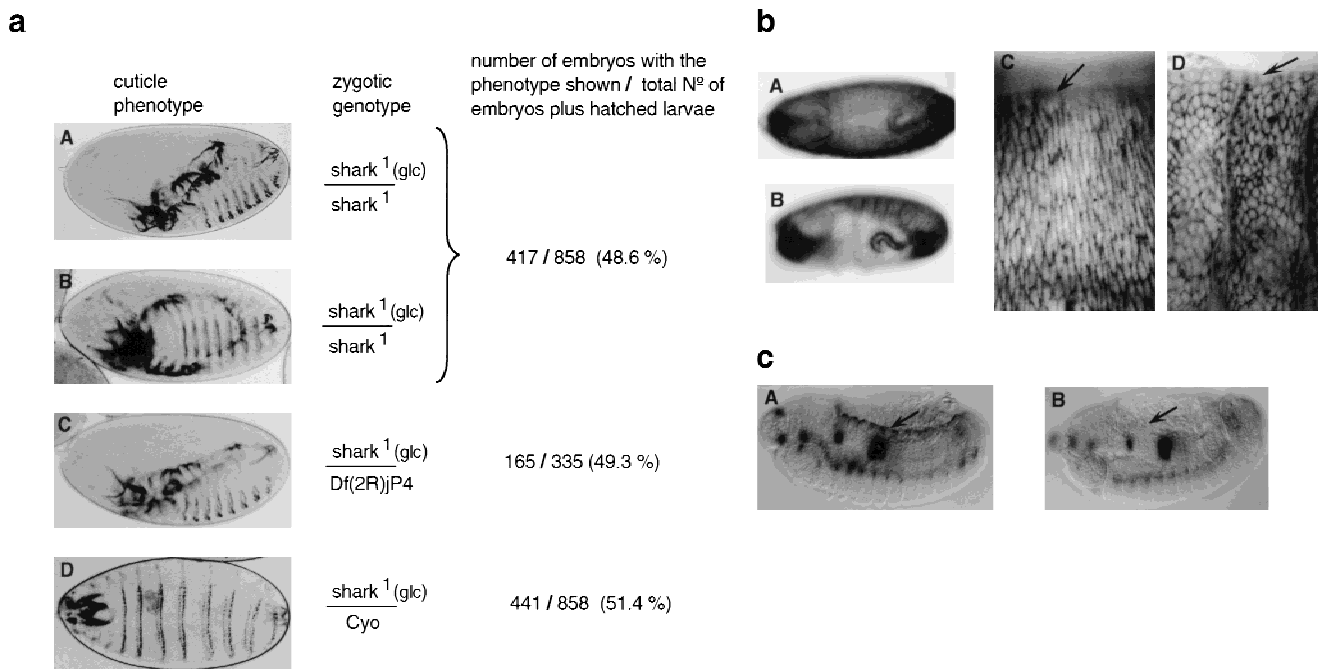
Figure 4. Phenotypic analysis of *shark¹* embryos lacking maternal and zygotic Shark activity. (*a*) Cuticular phenotypes associated with complete loss of *shark* function (anterior to the *left*). (*A,B*) Lateral and ventral views, respectively, of representative cuticles derived from *shark¹* GLCs 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*(glc)/*shark¹* (maternally derived chromosome/paternally-derived chromosome), with the embryonic lethal phenotype shown in *A* and *B* and their fully paternally rescued siblings, *shark¹*(glc)/*CyO*, shown in *D*]. (*C*) *shark¹* GLCs fertilized with *Df(2R)Jp7* 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 *cis* to *shark¹*. [In this case, females of the genotype *FRT42B, ovoD1/FRT42B, shark¹* were crossed to *Df(2R)Jp7/CyO*-balanced males, yielding two different zygotic genotypes: *shark¹*(glc)/*Df(2R)Jp7*, with phenotypes indistinguishable from those in *A* and *B* and the paternally rescued siblings, *shark¹*(glc)/*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)Jp7* 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* GLC fertilized with sperm carrying the *shark¹* allele, expressing UAS–*shark* 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*(glc)] 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.
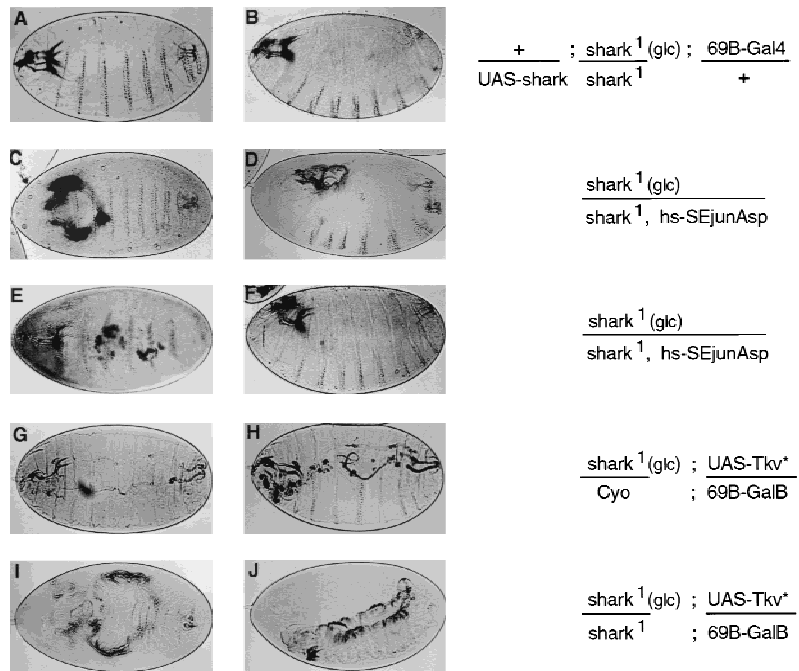
tent with the action of Shark upstream of *bsk* (JNK) in the JNK pathway in LE cells.

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–Pnt 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-

ered. 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 *shark¹* rescued females also showed reduced Shark accumulation in developing eggs as evidenced by the production of *shark¹* 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 *shark¹* 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 (Noselli 1998), *shark¹* phenotypes are clearly restricted to cell migration and the stretching of ectodermal cell sheets. However, it remains possible that further reduction in Shark expression, below levels in the developing *shark¹* pupae, would lead to effects in other structures.

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 juxtaposing dorsal mesoderm (Xu et al. 1998). In addition to their 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* c-*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

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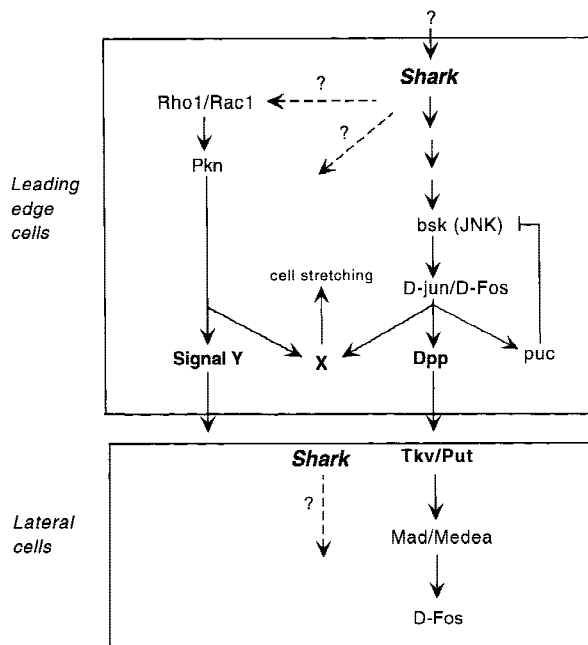
Figure 6. Schematic representation of Shark function in DC. The scheme summarizes our results, which indicate that Shark functions upstream of the JNK pathway in the LE, to regulate Dpp expression. The scheme also includes a JNK-independent pathway required in the LE that was proposed by Lu and Settleman (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 *Bgl*II site of the transformation vector pCasper-hs (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 analyses were performed as described (Chou et al. 1993; Chou and Perrimon 1996). The *shark*[1] allele was recombined into a chromosome carrying the FRT42B(mw⁺) transposon (Xu and Rubin 1993; Chou and Perrimon 1996). Heat shock (two 1-hr 37°C pulses at 48 and 72 hr after egg laying)-mediated recombination and generation of GLCs was carried out by crossing females of the genotype hs-FLP7; FRT42B, shark[1]/CyO to w; FRT42B, ovo[D1]/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′-CCCAAAAAGGATCCGCGATAGTGAT-CCCATG-3′ and 5′-CATAAATAGCTCGAGAAAATTGCAC-TCAGC-3′. PCR reactions were carried out with *Pfu* polymerase (STRATAGENE), digested with *Nco*I and *Not*I, and subcloned into the plasmid pBP4 (Driever et al. 1990). The insert was digested with *Bgl*II and subcloned into the expression vectors pCaSpeR-hs and pUAST. Functionality of these constructs and the loss of catalytic activity of the K698R 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 antisera 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 balancer chromosome containing a *lacZ* transgene (CyO, eve–lacZ, or TM6,Tb, ubx–lacZ) were purchased from Sigma (mouse polyclonal, diluted 1:1000) or 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 antibodies, 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 Axiophot) 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 Photoshop 4.0 for Macintosh. Composite figures were prepared with Adobe Illustrator 6.0.

In situ hybridizations

Third instar larva polytene chromosome squashes were prepared according to Pardue (1986) and probed by in situ hybridization with 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 digoxygenin-labeled RNA probes prepared by in vitro transcription (Boehringer Mannheim).

Molecular characterization of the shark[1] *allele*

shark[1] 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; shark[1]/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-ACGCAGCCAGGATCGGAGCTCATGGCCC-3′; primer 2, 5′-GAATGGTAACAAGGTGCAGTCGG-3′; primer 3, 5′-CG-

GTGGCAGGTCAATCTCAATGG-3′; and primer 4, 5′-GC-GGGATCCGATCTGTGGGTTCCAGATCGTTAATACG-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.

Acknowledgments

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Erratum

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