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FAST TRACK

Embryonic Morphogenesis Signaling Pathway Mediated by JNK Targets the Transcription Factor JUN and the TGF-β Homologue *decapentaplegic*

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Abstract The dorsal surface of the *Drosophila* embryo is formed by the migration of the lateral epithelial cells to cover the amnioserosa. The *Drosophila* cJun-N-terminal kinase (DJNK) is essential for this process. Mutations in DJNK or the DJNK activator *hemipterous* (HEP) lead to incomplete dorsal closure, resulting in a hole in the dorsal cuticle. The molecules downstream of DJNK in this signaling pathway have not been established. Here we demonstrate that the *basket1* (*bsk1*) mutation of DJNK causes decreased interaction with DJUN. Expression of *decapentaplegic* (DPP), a TGF-β homologue, in the leading edge of the dorsal epithelium, is identified as a genetic target of the JNK pathway. A constitutive allele of JUN is able to rescue the dorsal closure defect of *bsk1* and restores DPP expression. Furthermore, ectopic DPP rescues the defects in dorsal closure caused by *bsk1.* These data indicate that the interaction of DJNK with DJUN contributes to the dorsal closure signaling pathway and targets DPP expression. J. Cell. Biochem. 67:1–12, 1997.

Key words: DPP; *Drosophila;* mutations; dorsal closure signaling pathway; JNK pathway

The MAP kinase group of protein kinases is implicated in the regulation of many cellular processes, such as differentiation, cell growth, and the cellular response to the outside environment [Whitmarsh and Davis, 1996]. One distinct subgroup of MAP kinases are the c-Jun N-terminal kinases (JNK) [Whitmarsh and Davis, 1996]. The JNK MAP kinases are phosphorylated and activated by a MAP kinase kinase [Dérijard et al., 1995; Lin et al., 1995; Sanchez et al., 1994; Tournier et al., 1997] by dual phosphorylation on the motif Thr-Pro-Tyr in kinase subdomain VIII [Dérijard et al., 1994]. JNK is activated in response to numerous extracellular stimuli, including cytokines and environmental stress [Dérijard et al., 1994; Kyriakis et al., 1994].

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Substrates of the JNK protein kinase include the transcription factors c-Jun and ATF2 [Dérijard et al., 1994; Gupta et al., 1995; Kyriakis et al., 1994; Livingstone et al., 1995; van Dam et al., 1995]. The sites of phosphorylation by JNK are located in the NH_2-terminal activation domains of these transcription factors [Whitmarsh and Davis, 1996]. Recognition of these substrates by JNK requires a binding site that is separate from the sites of phosphorylation [Adler et al., 1992, 1994; Dai et al., 1995; Dérijard et al., 1994; Gupta et al., 1996; Hibi et al., 1993]. Analysis of the JNK sequences required for this binding interaction has identified a region of the kinase domain that confers high-affinity binding to c-Jun and ATF2 [Dai et al., 1995; Gupta et al., 1996; Kallunki et al., 1994; Sluss et al., 1994]. This region is alternatively spliced in mammalian JNK to create a group of ten isoforms that differ in their affinity for interaction with substrates [Gupta et al., 1996].

The AP-1 transcription factor has been identified as a target of the mammalian JNK signaling pathway [Whitmarsh and Davis, 1996]. Both

c-JUN and ATF-2 are members of the bZIP group of transcription factors that bind as homodimeric and heterodimeric complexes to AP-1 and AP-1-like sites in the promoters of many genes [Whitmarsh and Davis, 1996]. In vitro studies indicate that the phosphorylation of c-Jun and ATF-2 by JNK causes increased transcriptional activity [Whitmarsh and Davis, 1996]. This conclusion has been confirmed by gene disruption studies in mice demonstrating that the JNK signaling pathway is required for AP-1 transcriptional regulation in vivo [Yang et al., 1997]. It is therefore established that the JNK signaling pathway controls gene expression, in part, by the regulation of AP-1 transcriptional activity. However, the precise physiological function of mammalian JNK remains unclear [Whitmarsh and Davis, 1996].

To gain insight into the physiological function of JNK, we have investigated the role of JNK in the genetically tractable model organism, *Drosophila* [Sluss et al., 1996]. We and others have identified a *Drosophila* homologue of the mammalian c-Jun N-terminal kinase, DJNK [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. Flies with loss-of-function alleles of DJNK are not viable [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. DJNK is required for the morphogenetic process of dorsal closure [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. Dorsal closure occurs during mid-embryogenesis when the lateral epidermal cells elongate and spread over the amnioserosa to cover the dorsal surface of the embryo [Young et al., 1993]. The absence of DJNK blocks the epithelial cell elongation and migration [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. The DJNK protein kinase is therefore required for a signal transduction pathway that regulates embryonic morphogenesis. This signal transduction pathway may include a *Drosophila* Rho-family GTPase [Harden et al., 1995; Strutt et al., 1997], a *Drosophila* PAK protein kinase [Harden et al., 1996], the MAP kinase kinase HEP [Glise et al., 1995], and the MAP kinase DJNK [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. Biochemical studies demonstrate that DJNK is activated by HEP [Sluss et al., 1996], but the direct biochemical relationship between the other proposed components of this MAP kinase signaling pathway remains to be established.

The targets of the DJNK signaling pathway that are required for dorsal closure have not been identified. One possibility is that DJNK modifies pre-existing cellular components that regulate the cell shape change and movements, for example cell adhesion molecules and the cytoskeleton. Indeed, it has been reported that the accumulation of actin and non-muscle myosin is induced in the epithelial cells at an early stage of dorsal closure [Harden et al., 1995; Young et al., 1993]. In addition, genetic evidence has indicated that the non-muscle myosin heavy chain gene *zipper* [Young et al., 1993], the β-integrin homologue *l(1)myospheroid* [MacKrell et al., 1988], and the septate junction protein encoded by *coracle* [Fehon et al., 1994] are required for dorsal closure. Each of these genes represents a potential target of the DJNK signaling pathway.

An alternative mechanism of DJNK signaling is the induction of the expression of genes that are required for dorsal closure. Several genes have been identified that function early in development. Examples include *pannier* and *crumbs,* which contribute to the formation of the amnioserosa [Jurgens et al., 1984; Ramain et al., 1993; Winick et al., 1993] and other genes required for early dorsal-ventral patterning of the embryo. This latter group of genes includes members of a TGF-β signaling pathway, such as *dpp, schnurri, thick veins, saxophone,* and *punt* [Affolter et al., 1994; Arora et al., 1995; Brummel et al., 1994; Grieder et al., 1995; Ruberte et al., 1995; Twombly et al., 1996]. This DPP pathway, in addition to an early role in dorsoventral patterning, has also been implicated to function during the process of dorsal closure. For example, the *thick veins* mutation in the DPP receptor causes a hole in the dorsal region of the cuticle that may be caused by incomplete dorsal closure [Affolter et al., 1994; Letsou et al., 1995; Ruberte et al., 1995]. These data establish that the DPP signaling pathway is a potential target of DJNK during dorsal closure.

The purpose of this study was to examine the biochemical defect caused by the *bsk1* mutation in DJNK. The mutated DJNK^{bsk1} protein was found to be defective in interactions with its substrate DJUN, but not in interactions with its activator HEP. Genetic rescue experiments demonstrated that a constitutively activated allele of JUN complemented the dorsal closure defect caused by *bsk1.* The constitutively activated allele of JUN also restored a defect in the expression of DPP in the leading edge of the dorsal epithelium caused by *bsk1.* Ectopic ex-

pression of DPP rescued the defect in dorsal closure caused by *bsk1*. These data indicate that the TGF-β homologue DPP is a genetic target of the DJNK signaling pathway mediated by the transcription factor DJUN.

MATERIALS AND METHODS

Drosophila Strains

The wild-type *Drosophila* strain was Canton S. (CS). The *bsk1/CyO* line and DJNK deletion lines have been reported [Sluss et al., 1996]. The hsDPP line was kindly provided by Dr. S. Newfeld and W. Gelbart [Twombly et al., 1996]. P-element mediated transformation was done [Sluss et al., 1996] using the helper plasmid turbo Δ2-3 and the plasmid pCasperhsp70 containing c-JUNasp or c-JUNwt. The c-JUNwt allele [Treier et al., 1995] was subcloned from the pMT35 plasmid (kindly provided by Dr. D. Bohmann) in the pCasperhsp70 at the *Sac*I and *Hind*III sites. The cJUNasp allele [Treier et al., 1995] was subcloned from the plasmid pCasper3UASJUNasp in the pCasperhsp70 vector at the *Not*I and *Kpn*I sites. Two thousand embryos were injected with the hsJUNasp construct and 1,000 with the hsJunwt construct. Two independent transgenes were obtained with hsJUNwt and one line with hsJUNasp. The transgenes on the third chromosome were balanced and crossed [Sluss et al., 1996] to the *bsk1/CyO* line to generate a stock with the genotype *bsk1/CyO;hsJUN/TM6y+*. The hsDPP line [Twombly et al., 1996] was balanced and crossed [Sluss et al., 1996] to generate a line with *bsk1/CyO* and four copies of DPP.

Genetic Rescue Experiments and Embryo Staining

Embryos were collected at (0- to 3-h intervals) and aged to the appropriate time. Heat shock (1 h, 37°C) was done on embryos aged 6–9 h. The embryos were either prepared for RNA in situ hybridization or collected for cuticle analysis [Sluss et al., 1996]. To determine the loss of DPP expression, collections of embryos were aged until 6–9 h, treated or not with heat shock, and harvested for RNA in situ analysis using a DPP anti-sense riboprobe [Ray et al., 1991]. The presence or absence of DPP expression in the dorsal rim of the lateral epithelial cells was examined in embryos undergoing dorsal closure. If 15% of the embryos had lost DPP dorsal rim expression, the line was scored as having defective DPP expression. Cuticles (500) were analyzed for the presence or absence of the basket phenotype as described [Sluss et al., 1996]. The basket phenotype is the presence of a hole in the dorsal region in the embryo.

Immunohistochemistry

Embryos were dechorionated, fixed, and blocked (1 h) in a solution of PBS-T [Sluss et al., 1996] with 3% bovine serum albumin (BSA). Antibody staining was performed using a monoclonal antibody to JNK (Pharmingen, San Diego, CA) at 1:200 in PBS-T. The secondary antibody was an anti-mouse Ig antibody and immunodetection was done with the Vectastain kit (Vector Laboratories). The embryo staining and cuticles were examined with a Zeiss Axiophot Microscope. Photographs were taken using differential interference contrast optics.

Recombinant Proteins

Mutations at codon 225 in DJNK were generated by overlap extension polymerase chain reaction (PCR), cloned into the plasmid pCDNA3 (Invitrogen, Carlsbad, CA), and sequenced with an Applied Biosystems model 373A machine. The JNK and JNK^{bsk1} cDNAs were sub-cloned into the pGSTag vector at the *Bam*HI and *Sac*1 sites [Sluss et al., 1996]. The GST-JNK and GST-JNK^{bsk1} fusion proteins were purified by affinity chromatography, as described [Sluss et al., 1996]. The GST-DJUN and GST-HEP fusion proteins have been described previously [Sluss et al., 1996].

Kinase Assays

JNK activity was measured using protein extracts prepared from staged embryos harvested in Triton lysis buffer [Sluss et al., 1996]. Protein concentration was measured using the BCA assay kit (Pierce Chemical Co., Rockford, IL). Extracts (500 µg) were incubated (2 hr, 4°C) with GST-DJUN prebound to glutathione-agarose beads (Pharmacia LKB, Piscataway, NJ). The complexes were washed three times in Triton lysis buffer and once with kinase buffer [Sluss et al., 1996]. Assays were performed in 25 µl of kinase buffer with 20 µM [γ-^{32}P]ATP (10 Ci/mmol). The reaction was terminated after 10 min, and the products were examined by polyacrylamide gel electrophoresis (PAGE) and autoradiography. Kinase assays using recombinant proteins was done, as described [Sluss et al., 1996].

Binding Assay

Wild-type DJNK and mutant DJNK cDNAs cloned in the vector pCDNA3 (Invitrogen Inc.) were translated using the TNT® translation kit (Promega, Madison, WI) in the presence of ^{35}S-methionine (NEN-Dupont). The ^{35}S-labeled proteins were incubated in Triton lysis buffer [Sluss et al., 1996] that contained GST (5 µg) or GST-fusion proteins (5 µg) prebound to glutathione-agarose beads [Sluss et al., 1994]. The complexes were washed four times in Hepes-binding buffer [HB; 20 mM Hepes (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 25 mM $MgCl_2$, and 0.05% Triton X-100] and then examined by SDS–PAGE, and stained with Coomassie-blue [Sluss et al., 1994]. The DJNK proteins were detected by autoradiography and quantitated by Phosphorimager analysis [Sluss et al., 1994].

RESULTS

Biochemical Properties of DJNK

Mutations in the DJNK gene correspond to the complementation group basket [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. The *basket1* (*bsk1*) allele has a point mutation in codon 225; GGA (Gly) is replaced with GAA (Glu) [Sluss et al., 1996]. This residue is located in a region that is conserved in mammalian JNK and has been implicated in substrate recognition [Dai et al., 1995; Gupta et al., 1996; Kallunki et al., 1994; Sluss et al., 1994]. These data indicate that the loss-of-function mutation *bsk1* may cause defects in the interaction of DJNK with substrates. To test this hypothesis, we examined the interaction of DJNK with substrates.

We have previously demonstrated that DJUN, c-JUN, and ATF2 are substrates for DJNK [Sluss et al., 1996]. We therefore tested whether these proteins bind to DJNK in an in vitro binding assay. Figure 1A shows that DJNK binds to DJUN (lane 2), c-Jun (lane 3), and ATF2 (lane 4). We also tested whether the MAP kinase kinase HEP, which phosphorylates and activates DJNK [Sluss et al., 1996], binds to DJNK. In vitro binding assays confirmed that HEP binds to DJNK (Fig. 1A, lane 5). Comparative studies were performed using DJNK^{bsk1}. These experiments demonstrated that the *bsk1* mutation caused decreased binding of DJNK to the substrate DJUN (Fig. 1A, lane 7) without altering the interaction of DJNK with its activator HEP (lane 8).

To further examine the *bsk1* mutation (Gly-225 replaced with Glu), we investigated the effect of substitution of Gly-225 with different amino acids. Equal amounts of DJNK and the DJNK mutants were prepared by in vitro translation (Figure 1B). The binding of each DJNK mutant to the substrate DJUN was decreased compared to wild-type DJNK (Fig. 1C). The loss-of-function phenotype caused by the *bsk1* mutation therefore results from the loss of Gly-225, rather than the replacement with Glu. These data suggest that Gly-225 is critical for the creation of the DJNK surface that is involved in substrate recognition.

Previous studies have demonstrated that substrate binding is required for efficient phosphorylation of substrate proteins by JNK [Adler et al., 1992, 1994; Dai et al., 1995; Dérijard et al., 1994; Gupta et al., 1996; Hibi et al., 1993]. The *bsk1* mutation is therefore predicted to cause decreased substrate phosphorylation by DJNK. To test this hypothesis, we examined the protein kinase activity of DJNK and DJNK^{bsk1} in an in vitro protein kinase assay. The DJNK activator HEP did not phosphorylate DJUN (Fig. 2, lane 1), but HEP was found to phosphorylate DJNK and cause a large increase in DJUN phosphorylation by DJNK (lane 3). Comparative studies using JNK^{bsk1} demonstrated that this mutated protein kinase did not phosphorylate DJUN (lanes 5 and 6). However, JNK^{bsk1} was a substrate for HEP (lane 6). These data demonstrate that the *bsk1* mutation selectively blocks the phosphorylation of substrates by DJNK without affecting the phosphorylation of DJNK by its activator HEP. It is likely that the loss of substrate phosphorylation by DJNK accounts for the loss-of-function phenotype of *bsk1*.

JNK Protein Kinase Activity Correlates With Dorsal Closure

The expression of DJUN is ubiquitous in the ectoderm, including the lateral epithelial cells [Perkins et al., 1988, 1990; Zhang et al., 1990]. The DJNK protein kinase is also localized in the lateral epithelial cells (Fig. 3A). The co-localization of DJNK with DJUN in the lateral epithelial cells suggests that DJUN may be a physiologically relevant target of the DJNK signaling pathway during dorsal closure. This possibility requires that DJNK is active during dorsal closure. To test this hypothesis, we prepared extracts from staged embryos and mea-

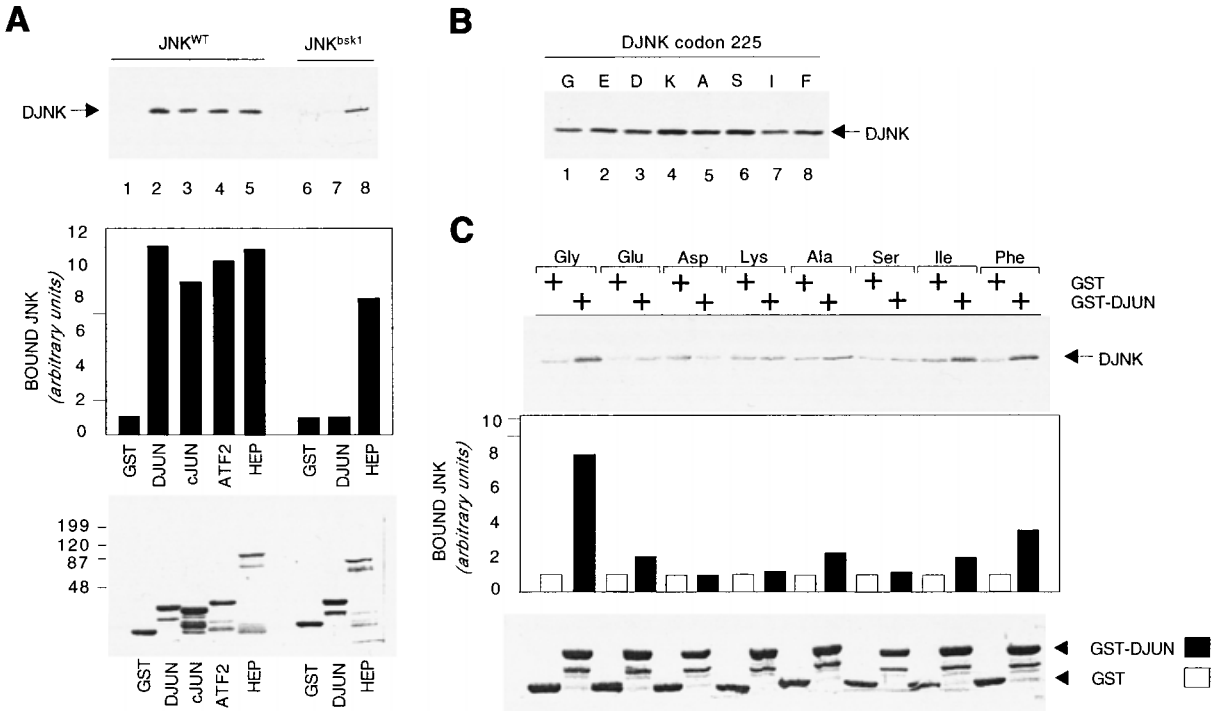

Fig. 1. Comparison of the biochemical properties of wild-type and *bsk1* allele of JNK. **A:** *Top:* Binding of in vitro translated DJNK wild-type protein to GST (**lane 1**), GST-DJUN (**lane 2**), GST-cJUN (**lane 4**), and GST-HEP (**lane 5**); and binding of JNK^{bsk1} to GST (**lane 6**), GST-DJUN (**lane 7**) and GST-HEP (**lane 8**). *Middle:* The binding data were quantitated by Phosphorimager analysis. Similar results were obtained in two experiments. *Bottom:* Coomassie-blue stained gel of the experiment shown in A. The numbers on the left represent protein mass markers in kilodaltons. **B:** Autoradiography of an SDS–PAGE gel showing the translated products wild-type DJNK G225 (**lane 1**), G225E (**lane 2**), G225D (**lane 3**), G225K (**lane 4**), G225A (**lane 5**), G225S (**lane 6**), G225I (**lane 7**), G225F (**lane 8**). **C:** *Top:* Binding of wild-type DJNK (Gly225) and various mutations of DJNK at codon (Glu, Asp, Lys, Ala, Ser, Ile, Phe). *Middle:* Coomassie-blue stained gel of proteins. *Bottom:* The binding data were quantitated by Phosphorimager analysis. Similar results were obtained in two other experiments.

sured DJNK protein kinase activity [Sluss et al., 1996]. The DJNK protein kinase activity was first detected after 6–8 hours of development and persisted throughout the stages of dorsal closure (Fig. 3B). The correlation between increased DJNK protein kinase activity and the timing of dorsal closure indicates that substrate phosphorylation by DJNK may play a role in dorsal closure. The DJUN protein represents one candidate target of the DJNK pathway in this process.

Constitutively Activated JUN Complements Loss-of-Function Alleles of DJNK

We performed a genetic rescue experiment to test the hypothesis DJUN is relevant to the process of dorsal closure. The rationale for this experiment was that if the activation of DJUN by DJNK was required for dorsal closure, a constitutively activated allele of DJUN should by-pass the requirement for DJNK. A constitutively activated JUN allele, in which the activating phosphorylation sites are replaced with Asp (JUNasp), has been extensively characterized [Treier et al., 1995]. We generated transgenic flies with JUNasp and wild-type JUN (JUNwt) under the control of the heat-shock promoter. These transgenic flies were crossed to obtain lines with the genotype *bsk1/CyO; hsJUN*. We then tested whether the defect of dorsal closure could be rescued by the expression of ectopic JUN.

The embryos with defects in dorsal closure have a hole (either severe or moderate) in the dorsal region of the cuticle, the basket phenotype. Mendelian inheritance indicates that the expected number of basket cuticles is 25%. Heat shock of the *hsJUNwt* or *hsJUNasp* parental transgenic flies did not induce dorsal hole phenotypes (data not shown). The number of basket cuticles from *bsk1/CyO; hsJUNwt* embryos remained the same in the absence or presence of heat-shock treatment (data not shown). Therefore, the wild-type JUN allele did not

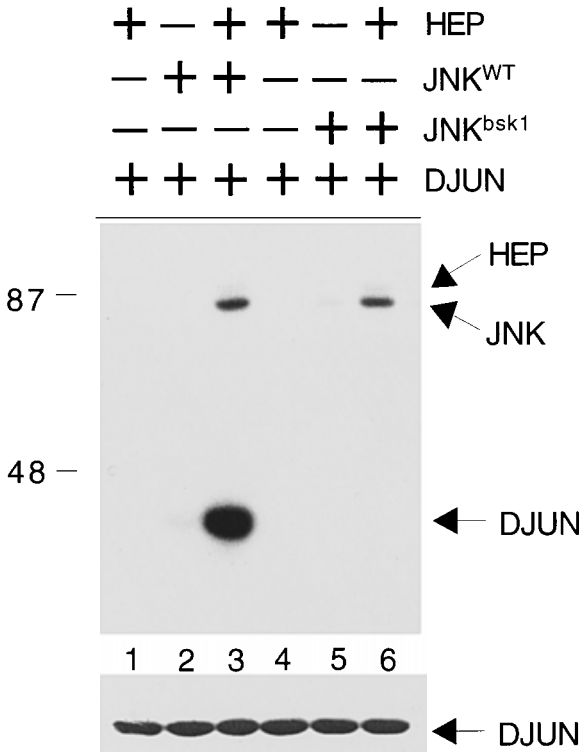

Fig. 2. Biochemical properties of wild-type and *bsk1* allele of JNK. Comparison of kinase activity of wild-type DJNK (G225) and JNK^{bsk1}. *Top:* Recombinant DJNK (**lanes 2–3**) or JNK^{bsk1} (**lanes 5, 6**) was used to phosphorylate GST-DJUN in the absence (**lane 2, 5**) and presence (**lane 3, 6**) of GST-HEP. GST-HEP alone was used to phosphorylate GST-DJUN (**lanes 1, 4**). The samples were examined by SDS–PAGE and autoradiography. The number on the left represents protein mass markers in kilodaltons. *Bottom:* Coomassie-blue-stained gel of the experiment shown at top.

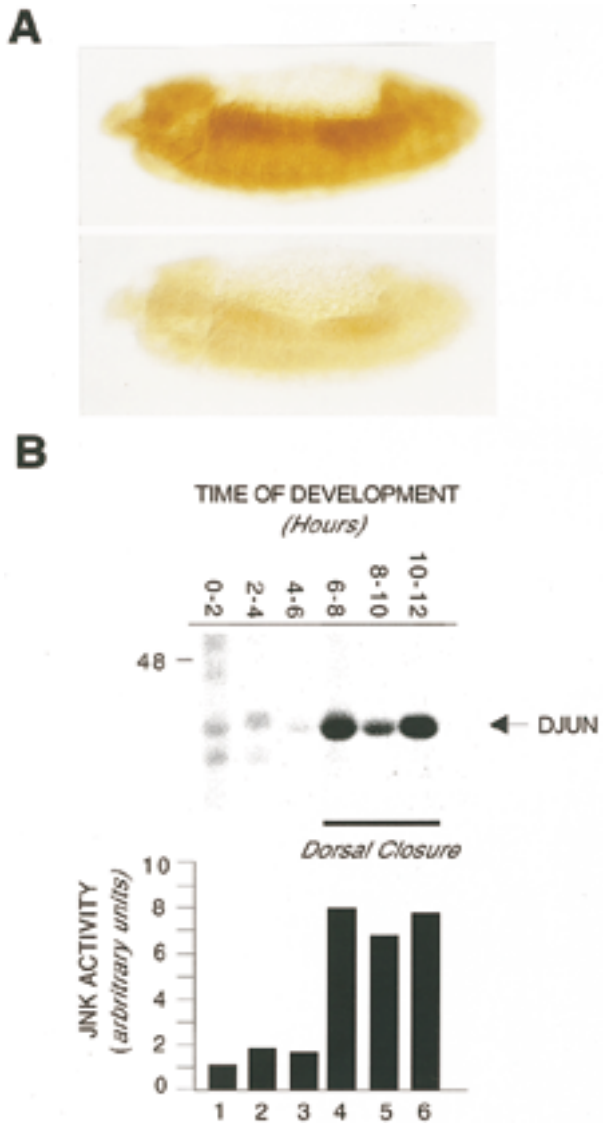

Fig. 3. JNK protein kinase activity during development. **A:** Whole-mount embryos at stage 12 stained with a JNK monoclonal antibody. The orientation is such that anterior is left and dorsal is up. The focal plane is on the lateral epidermal cells. *Top:* Wild-type embryo. *Bottom:* DJNK −/− embryo (*flp170b*) [Sluss et al., 1996]. **B:** Time course of DJNK protein kinase activity during development. The bar indicates the time when dorsal closure occurs. Embryos were collected (0–2 h) and aged until the indicated times. The DJNK protein kinase activity in the embryos was examined in extracts using the substrate DJUN. *Top:* The samples were examined by SDS–PAGE and autoradiography. *Bottom:* The DJNK protein kinase activity was quantitated by Phosphorimager analysis. The data presented are the mean of two separate experiments.

rescue the defects in dorsal closure caused by *bsk1*. By contrast, the constitutively-activated JUN allele did rescue the basket phenotype (Fig. 4D). The number of dead embryos did not change, but the number of basket cuticles was reduced from 19% to 2% with heat-shock (Fig. 4C,D). The cuticles of the dead embryos had either a plug in the dorsal surface (Fig. 4D) or were wild-type in appearance (data not shown), similar to the rescue of *bsk1* with hsJNK (Fig. 4B). Control experiments demonstrated that heat shock of the parental *bsk1* or JNK (−/−) lines (Sluss et al., 1996) did not alter the number of basket cuticles (data not shown).

Together, these data demonstrate that a constitutively activated allele of JUN rescues the defect in dorsal closure caused by loss-of-function alleles of DJNK (Fig. 4C,D). Furthermore, a DJNK allele (*bsk1*), which has lost the ability to bind and phosphorylate DJUN (Fig. 1), can be complemented by an allele of JUN that mimics phosphorylation and does not require interaction with DJNK. These data provide genetic evidence that DJUN is a relevant target of DJNK during dorsal closure.

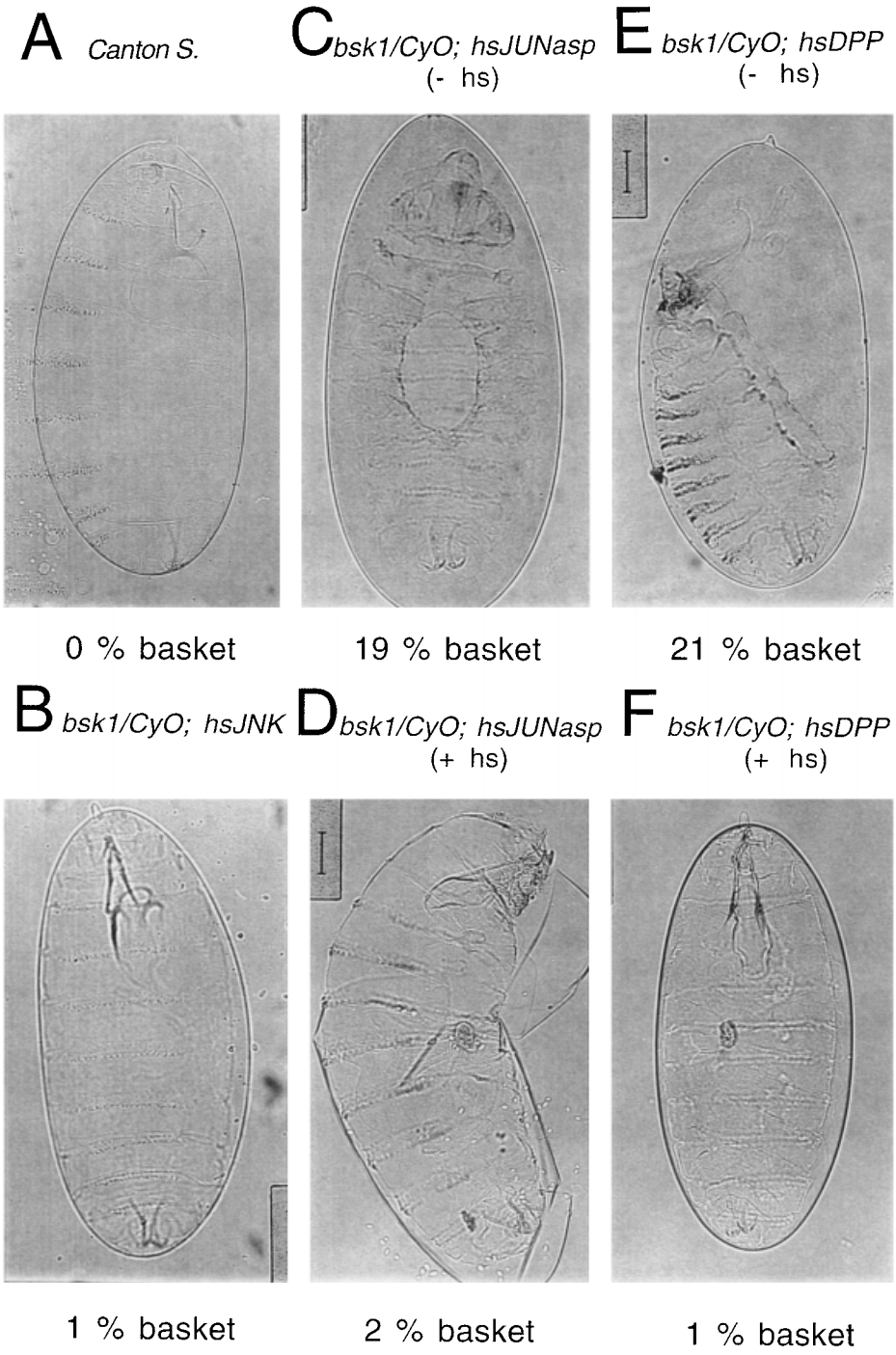

Fig. 4. Rescue of dorsal closure defect by ectopic JUN and DPP. The genotypes of the flies used for embryo collection is noted above **A–F**. The numbers below each panel represent the percentage of basket cuticles from the population of collected embryos. Embryos (500) were collected from the lines and aged. The number of dead embryos was determined and cuticles examined for the basket phenotype, defined as either a severe (**E**) or moderate (**C**) hole in the dorsal cuticle [Sluss et al., 1996]. The orientation is such that anterior is up. **A:** Wild-type cuticle illustrating the regular spacing of the denticle belt on the ventral side (*left*) and complete closure of the epidermis on the dorsal region (*right*). **B:** Cuticle of a *bsk1/bsk1; hsJNK* embryo illustrating complete rescue for the defect in dorsal closure. **C:** Cuticle of a *bsk1/bsk1; hsJUNasp* embryo without heat shock, illustrating a moderate defect in dorsal closure. **D:** Cuticle of a *bsk1/bsk1; hsJUNasp* embryo with heat shock, demonstrating the rescue of dorsal closure. **E:** Cuticle of a *bsk1/bsk1; hsDPP* embryo illustrating a severe defect in dorsal closure. **F:** Cuticle of a *bsk1/bsk1; hsDPP* embryo with heat-shock in which the process of dorsal closure has been completed, except for a minute region in the dorsal surface.

The JNK Pathway Is Linked to DPP Signaling

DPP can function to induce cell migration [Twombly et al., 1996] and signal to adjacent cells [Hursch et al., 1993; Immerglück et al., 1990; Panganiban et al., 1990; Reuter et al., 1990; Twombly et al., 1996]. This observation suggests that there could be a role for DPP in the spreading of the dorsal epithelium. Indeed, the DPP signaling pathway has been genetically implicated to function in dorsal closure [Affolter et al., 1994; Letsou et al., 1995; Ruberte et al., 1995]. DPP expression is not ubiquitous during embryogenesis. In the stages of dorsal closure, the expression of DPP includes two lateral stripes in the epithelium [Jackson and Hoffmann, 1994]. The upper lateral stripe of DPP expression is in the rim of cells adjacent to the amnioserosa, the dorsal rim [Irish and Gelbart, 1987; Jackson and Hoffmann, 1994]. These dorsal rim cells are postulated to play an important role in the cell shape change that occurs prior to spreading [Young et al., 1993]. These data suggest that DPP could be a signal for cell movement in the epithelium during dorsal closure and may therefore be a target of the JNK pathway. To test this hypothesis, we examined the expression pattern of DPP in wild-type and DJNK mutant embryos. Examination of embryos from the *bsk1/CyO* line demonstrated that DJNK is not required for DPP expression in the blastoderm or in the ectoderm prior to dorsal closure (Fig. 5A–D). Embryos in stages during dorsal closure [Jackson and Hoffmann, 1994] showed DPP staining in the hindgut, clypeolabrum, pharynx, esophagus, and parasegment 7 (Fig. 5C–F). The lower lateral stripe of DPP in the ectoderm is present (Fig. 5A–D). However, the upper DPP stripe in the dorsal rim of the ectoderm was absent or severely reduced in embryos during the stages of dorsal closure (Fig. 5D,F). This altered pattern DPP expression in the dorsal rim during dorsal closure was also detected in lines where the DJNK gene was deleted (data not shown). These data indicate that DPP expression in the dorsal rim requires DJNK.

Ectopic DPP Complements Loss-of-Function Alleles of DJNK

To investigate whether DPP expression in the dorsal rim is relevant to DJNK-mediated dorsal closure, we examined the effect of ectopic DPP on the defects associated with *bsk1*. We crossed a transgenic line expressing DPP under the control of the heat-shock promoter with the *bsk1/CyO* line. Embryos from the *bsk1/CyO; hsDPP* flies were collected and examined for viability and cuticle morphology (Fig. 4F). Without heat shock, the percentage of basket cuticles (21%) was similar to the *bsk1/CyO* line (Sluss et al., 1996). A pulse of heat shock (1 h) on embryos aged 6–9 h decreased the number of basket cuticles (1%), indicating that the DPP transgene rescued the dorsal closure defect caused by *bsk1*. Similar rescue by DPP was obtained in experiments using deletion lines of DJNK (data not shown). The number of dead embryos, however, did not change. The cuticles of the dead embryos were examined. A plug in the dorsal surface was found in some cuticles (Fig. 4F), and others appeared as wild-type (data not shown). Therefore, ectopic expression of DPP is able to rescue the defects in the dorsal

Fig. 5. Analysis of DPP expression in the leading edge of the dorsal epithelium. Whole mount RNA in situ of embryos stained with a DPP antisense riboprobe. The focal plane is on the lateral epidermal cells. A population of embryos from *bsk1/CyO* (**A–F**) or *bsk1/CyO; hsJUNasp* (**G,H**) flies were collected, aged until 6–9 h, and stained with a DPP antisense riboprobe. Representative embryos are shown. **A:** Wild-type early-stage 11 embryo. The orientation is such that anterior is left and dorsal is up. The germ band is not yet extended. DPP is expressed in two bilateral stripes in the ectoderm; an upper lateral stripe along the dorsal rim of epithelial cells and a lower lateral stripe, as well as other structures (the primordium of the pharynx, esophagus, clypeolabrum, and parasegment 7). **B:** *bsk1/bsk1* embryo in stage 11, demonstrating the DPP expression is the same as wild-type. **C:** Wild-type early-stage 12 embryo, in the process of dorsal closure. DPP is expressed in the two bilateral stripes in the ectoderm; DPP is also expressed in the clypeolabrum, pharynx, esophagus, gastric caeca, the hindgut, and parasegment 7. **D:** *bsk1/bsk1* embryo in early-stage 12, demonstrating severe reduction of DPP expression in the dorsal rim and no effect on other DPP expression. The orientation is such that anterior is left and dorsal is up. **E:** Dorsal view of a wild-type embryo in late stage 12. DPP is expressed in the two bilateral stripes in the ectoderm; DPP is also expressed in the hindgut and bilateral in the clypeolabrum, pharynx, esophagus, gastric caeca, and parasegment 7. Anterior is left. **F:** Dorsal view of a late-stage 12 *bsk1/bsk1* embryo. DPP expression is reduced in the dorsal rim, but present in the lower lateral stripe and expressed in the hindgut and bilateral in the clypeolabrum, pharynx, esophagus, gastric caeca, and parasegment 7. Anterior is left. **G:** Late-stage 12 *bsk1/bsk1; hsJUNasp* embryo that received heat shock. The orientation is such that anterior is left and dorsal is up. DPP expression is present as wild-type, including the dorsal rim expression. **H:** Late-stage 12 *bsk1/bsk1; hsJUNasp* embryo that did not receive heat shock. The orientation is such that anterior is left and dorsal is up. DPP expression is present in the lower lateral ectoderm stripe, but decreased in the upper lateral ectoderm stripe.

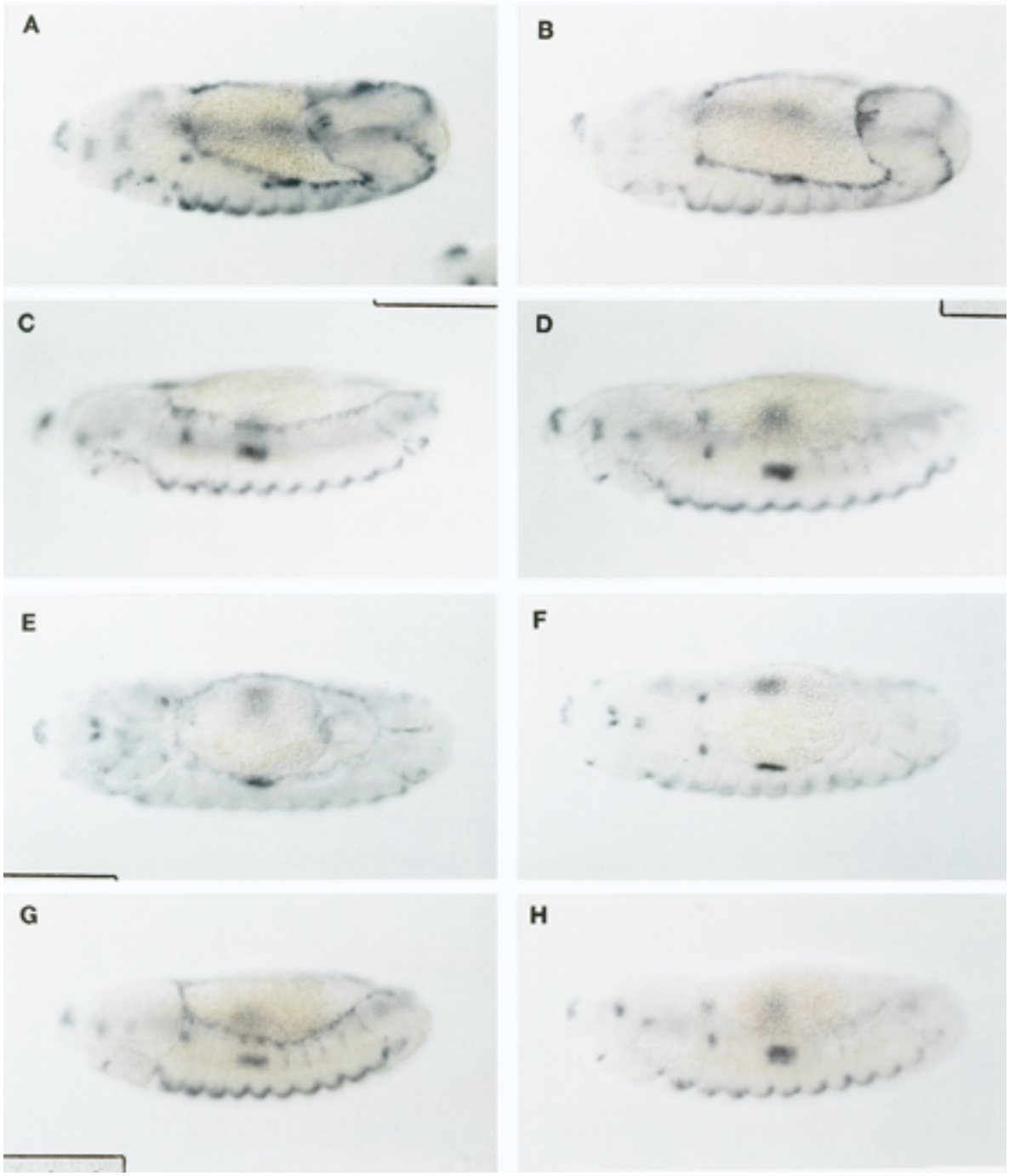

Figure 5.

epidermis caused by *bsk1*. These data suggest that DPP functions genetically downstream of DJNK.

DJNK Interaction With DJUN Regulates DPP Dorsal Rim Expression

We tested whether there is a link between DJUN and the DJNK-dependent expression of DPP in the dorsal rim by examining the effect of expression of a constitutive allele of JUN under the control of the heat-shock promoter. We examined DPP expression in embryos collected from the *bsk1/CyO; hsJUNasp* line. The embryos were stained with a DPP antisense riboprobe. In the absence of heat-shock, we observed embryos which lacked the dorsal rim expression of DPP (Fig. 5H). With heat-shock, all the embryos examined contained DPP expression in the dorsal rim (Fig. 5G). Control experiments demonstrated that heat-shock treatment of wild-type embryos did not alter DPP expression (data not shown). This result implies that DJUN may be responsible for direct or indirect regulation of the temporal expression of DPP in the dorsal rim.

DISCUSSION

The process of concerted cell movement, which is required for embryonic morphogenesis, can be genetically dissected using a tractable model system, such as *Drosophila*. Gastrulation represents one example of morphogenetic cell movements that have been studied in detail [Leptin, 1995]. A second example is the spreading of the dorsal epithelium (dorsal closure). The mechanisms that account for dorsal closure are poorly understood, but recent studies have led to elucidation of components of the signaling pathway that controls this morphogenetic process [Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996].

Dorsal closure is initiated during mid-embryogenesis when the lateral epithelial cells are triggered to elongate and then to spread and migrate to cover the amnioserosa [Ashburner, 1989]. The body plane of the *Drosophila* embryo is surrounded by epithelial cells and in the final process, the lateral cells are joined at the midline to complete the epithelium. The process of dorsal closure is regulated by a conserved MAP kinase signal transduction pathway which may include a *Drosophila* Rho-family GTPase [Harden et al., 1995; Strutt et al., 1997], a *Drosophila* PAK kinase [Harden et al., 1996], the MAP kinase kinase HEP [Glise et al., 1995], and the MAP kinase DJNK [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. It is expected that this pathway is linked to the molecules that direct the morphological changes and movement of the dorsal epithelial cells.

Loss-of-function alleles of DJNK correspond to the *basket* complementation group [Riesgo-Escovar et al., 1996; Sluss et al., 1996]. The *bsk1* mutation causes the replacement of Gly-225 with Glu [Sluss et al., 1996]. Biochemical analysis of this mutated DJNK indicated that it bound, and was phosphorylated by, its activator HEP (Fig. 1). However, $DJNK^{bsk1}$ was defective in binding and phosphorylation of its substrate DJUN (Fig. 1). These data identify the transcription factor DJUN as a target of DJNK signaling that may be relevant to the process of dorsal closure. Thus, the transcription factor DJUN may mediate signaling by the DJNK pathway. This hypothesis was tested by genetic epistatic analysis. These studies demonstrated that a constitutively activated JUN allele rescued the defect in dorsal closure caused by *bsk1* (Fig. 4). The regulation of DJUN by DJNK may therefore regulate the expression of genes that are required for dorsal closure.

The *decapentaplegic* (DPP) signaling pathway has been implicated in dorsal closure [Affolter et al., 1994; Letsou et al., 1995; Ruberte et al., 1995]. The cells that form the leading edge of the epithelium adjacent to the amnioserosa undergo elongation prior to spreading and are critical for the initiation of cell spreading during dorsal closure [Harden et al., 1995; Young et al., 1993]. DPP expression in the leading edge of the epithelium correlates with the movement and completion of dorsal closure [Jackson and Hoffmann, 1994]. Therefore, DPP expression is a likely candidate as a target of a dorsal closure signal transduction pathway. Furthermore, it is known that the DJNK pathway interacts genetically with the DPP because *bsk1* enhances a wing vein phenotype of DPP [Staehling-Hampton et al., 1995]. To identify whether DPP is a downstream target of the DJNK pathway, we examined the effect of *bsk* on the expression of DPP. The stripe of DPP expression corresponding to the dorsal rim of the epithelium was dependent on the JNK signal transduction pathway (Fig. 5). Expression of a constitutively activated allele of JUN restored this defect in DPP expression (Fig. 4). Furthermore, ectopic DPP expression rescued the dorsal closure defect caused by *bsk* (Fig. 5).

The results of the genetic epistatic analysis indicate that DJUN is a target of DJNK and that DPP is a target of both DJNK and DJUN. The biochemical basis of these interactions remains to be firmly established. However, one plausible hypothesis is that DJNK activates DJUN by phosphorylation and that the activated DJUN induces DPP expression. No mutations in the DPP promoter which specifically abolish dorsal rim expression have been identified [Jackson and Hoffmann, 1994]. It is therefore unclear whether DJUN induces DPP expression directly through a *cis*-acting element in the DPP promoter, whether DJUN acts in concert with other transcription factors, or whether the effects of DJUN are mediated by an indirect mechanism. In addition, the mechanism that restricts DPP expression to the dorsal rim of the epithelium is unclear. Further studies are therefore required to identify the biochemical basis of the DJNK–DJUN–DPP signaling pathway in the dorsal rim of the ectoderm.

The results of this study demonstrate that DJNK mutations cause defective DPP expression in the dorsal rim, leading to the dorsal open phenotype. It appears that the interaction of JNK with JUN is a primitive signaling module, at least in dorsal closure, which is used to coordinate a complex morphological process.

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