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Drosophila Jun relays the Jun amino-terminal kinase signal transduction pathway to the Decapentaplegic signal transduction pathway in regulating epithelial cell sheet movement

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We have characterized mutations in the *Drosophila* homolog of the mammalian proto-oncogene c-Jun gene (*Djun*). We demonstrate that DJUN in the embryo is a downstream target of the JNK signal transduction pathway during dorsal closure formation, and that the function of the JNK/DJUN pathway is to control the localized expression of *decapentalegic* (*dpp*), a member of the TGF-β growth factor family. In contrast to previous observations, we find that both in the embryo and during photoreceptor cell determination, DJUN is not regulated by a pathway that involves MAPK.

[*Key Words:* Jun; *Drosophila* Jun; JNK; Dpp; signal relay; dorsal closure]

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Investigations in mammalian cell systems have strongly suggested that the c-*jun* as well as c-*fos* genes have important roles in programs of cell growth and differentiation in which cells integrate external physiological signals to bring about appropriate transcriptional changes (Greenberg and Ziff 1984; Lee et al. 1987; Bartel et al. 1989; Ryder et al. 1989).

In *Drosophila*, only one *jun*- and one *fos*-related gene have been identified. *Drosophila* Jun and Fos exhibit biochemical similarity to their mammalian counterparts (Perkins et al. 1990; Zhang et al. 1990). Two different mitogen associated protein (MAP)-type kinases (MAPK), encoded by the *basket* (*bsk*) and *rolled* (*rl*) genes, are good candidates for kinases that phosphorylate and activate DJUN (Peverali et al. 1996; Riesgo-Escovar et al. 1996; Sluss et al. 1996). *bsk* encodes a homolog of mammalian Jun amino (N)-terminal kinase (JNK) (DJNK) (Riesgo-Escovar et al. 1996; Sluss et al. 1996). DJNK can efficiently phosphorylate DJUN in vitro, but it is not clear whether this occurs in vivo (Sluss et al. 1996). Furthermore, DJUN is phosphorylated directly by Rolled (Rl)/MAPK in vitro and this phosphorylation is enhanced in vivo when the gain-of-function Rl/MAPK *Seven maker* (rl^{Sem}) mutation is used (Peverali et al. 1996). Previous studies, using dominant negative and constitutively activated forms of *Djun*, have suggested that DJUN is a target of Rl/MAPK in Sevenless (Sev) receptor tyrosine kinase (RTK) pathway for differentiation of R7 photoreceptor cells (Bohmann et al. 1994; Treier et al. 1995; Peverali et al. 1996). To gain insights into the function of DJUN and to identify the signal transduction pathways that regulate the activity of DJUN in vivo, we have isolated mutations in *Djun* and analyzed their mutant phenotypes. Our analysis implicates that DJUN is required for a cell sheet movement during dorsal closure in a *Drosophila* embryo.

In a wild-type embryo, dorsal closure begins during mid-embryogenesis, takes ~2 hr (11–13 hr after egg laying: stage 13–15) (Campos-Ortega and Hartenstein 1985; Martinez Arias 1993), and involves cell shape change but no cell divisions. Before stage 13, all cells that comprise the ventral and lateral epidermis are polygonal in shape and become elongated after stage 15 (Young et al. 1993). Cell shape elongation along the dorsal–ventral axis starts from the dorsal-most cells of the epithelium and is driven by a structure at the dorsal side of the cells referred to as "the leading edge" during germ-band retraction. As dorsal closure proceeds, cell shape elongation is propagated to lateral and ventral epidermal cells. Dorsal closure is completed with the fusion at the dorsal mid-

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line of both edges and the internalization of the amnioserosa (Campos-Ortega and Hartenstein 1985; Martinez Arias 1993; Young et al. 1993).

A number of mutations in *Drosophila* have been identified that disrupt dorsal closure and display a common "dorsal open" phenotype (Jürgens et al. 1984; Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984; Perrimon et al. 1989). Some of the corresponding genes have been analyzed at the molecular level. These genes can be assigned to two main classes: genes coding for structure proteins required for dorsal closure, and genes involved in the regulation of dorsal closure.

The first class includes *zipper* (*zip*), which encodes a nonmuscle myosin heavy chain (Young et al. 1993); *inflated,* coding for an α-integrin subunit (Wilcox et al. 1989); *lethal(1)myospheroid,* coding for a β-integrin subunit (Mackrell et al. 1988); and *coracle,* which encodes a band 4.1 homolog (Fehon et al. 1994).

The second class of genes are most likely involved in triggering the dorsal closure process. These include members of the JNK and Decapentalegic (Dpp) signaling pathways. The JNK pathway is represented by *bsk* (DJNK; Riesgo-Escovar et al. 1996; Sluss et al. 1996), *hemipterous* (*hep*), which encodes the *Drosophila* JNK kinase (DJNKK; Glise et al. 1995), and *Drac,* which is a homolog of mammalian small GTPase Rac (Luo et al. 1994; Harden et al. 1995). Mutations in *bsk* and *hep* and expression of a dominant-negative form of *Drac* ($Drac^{DN}$) disrupt dorsal closure (Luo et al. 1994; Glise et al. 1995; Harden et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). The Dpp pathway is represented by the genes coding for Dpp receptors, *thick veins* and *punt,* as well as a downstream component encoded by the *schnurri* gene. Mutations in these genes also show dorsal open phenotypes (Affolter et al. 1994; Brummel et al. 1994; Nellen et al. 1994; Penton et al. 1994; Arora et al. 1995; Grieder et al. 1995; Letsou et al. 1995; Ruberte et al. 1995; Staehlong-Hampton et al. 1995).

We find that DJUN in vivo is a target of the JNK signal transduction pathway. Furthermore, we demonstrate that one of the functions of the JNK/DJUN pathways is to regulate the expression of *dpp* in the dorsal-most cells of the embryonic epidermis during dorsal closure. Finally, in contrast to previous observations (Bohmann et al. 1994; Treier et al. 1995; Peverali et al. 1996), we find that both in the embryo and during photoreceptor cell determination, DJUN is not regulated by a pathway that involves MAPK.

Results

Isolation of mutations in the Djun *locus*

Djun maps to 46E on the right arm of chromosome 2, a region saturated previously for lethal complementation groups by Bour et al. (1995). Southern blots of genomic DNA from the lines associated with lethality revealed that one of the zygotic lethal mutations, which we named $Djun^1$, identifies a deficiency of ~700 bp in the 2.4-kb *Eco*RI fragment of *Djun* genomic DNA (Fig. 1B).

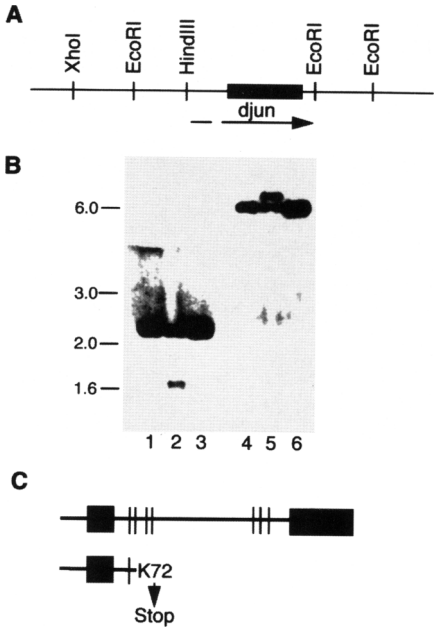

Figure 1. Molecular characterization of the *Djun* mutations. (*A*) Restriction map of the *Djun* gene (Wang and Goldstein 1994). (*B*) Southern hybridization analyses of *Djun* mutations. Genomic DNA was digested with restriction endonuclease *Eco*RI (lanes *1,2,3*) and *Hin*dIII (lanes *4,5,6*) and probed with *Djun* cDNA. DNA was prepared from flies of the following phenotypes. (Lanes *1,4*) Wild type; (lanes *2,5*) $Djun^1$/+; (lanes *3,6*) $Djun^2$/+. The position of DNA size markers in kilobases is indicated. (*C*) Schematic representation of wild-type and $Djun^2$ gene products. The Delta domain (square), the bZIP DNA-binding domain (rectangle), and phosphorylation sites (vertical bars), are shown (Hill and Treisman 1995). The amino acid exchange found in $Djun^2$ is indicated.

We found that $Djun^1$ failed to complement a mutation identified previously *l(2)IA109,* described by Nüsslein-Volhard et al. (1984), which we have renamed $Djun^2$. The following additional lines of evidence demonstrate that the two mutations are in the *Djun* gene. First, a P-element transformed line that includes a 5-kb *Djun* genomic DNA rescues the lethality of $Djun^1$ and $Djun^2$. Second, transient zygotic expression of a single copy of *Djun* cDNA under the heat shock promoter in $Djun^1$ homozygous mutant embryos is sufficient to restore the mutant embryos to a wild-type phenotype (data not shown). Third, repeated heat shock induction of a *hs–Djun* cDNA transgene during development was sufficient to rescue the lethality of transheterozygous $Djun^1$/$Djun^2$ animals. Finally, $Djun^2$ appears normal on Southern blots (Fig. 1B), but sequence analysis revealed that $Djun^2$ sequence has a stop codon at amino acid position 72, resulting in a predicted protein that terminates immediately after the amino-terminal "Delta" domain of the DJUN protein (Fig. 1C). Both $Djun^1$ and $Djun^2$ are likely to represent complete loss-of-function mutations in the *Djun* gene, as the embryonic phenotypes caused by $Djun^1$ *and* $Djun^2$ alleles when heterozygous to a de-

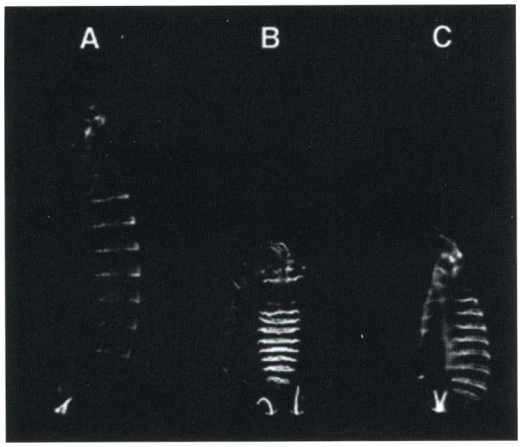

Figure 2. Cuticle phenotype of *Djun* embryos. (*A*) Dark-field cuticle pattern of a wild-type embryo. (*B*) $Djun^1/Djun^1$ embryo. (*C*) $Djun^2/Djun^2$ embryo. The embryos in *B* and *C* show similar dorsal open phenotypes represented by lack of thoracic and abdominal dorsal cuticle. *A* and *C* are lateral view. *B* is a ventral view.

letion are the same as those caused by the corresponding *Djun* homozygotes.

Djun *mutations disrupt the initiation of dorsal closure*

Djun mutant animals derived from heterozygous mothers die during embryogenesis. They display head defects and lack the dorsal epidermis that is detected by a large dorsal hole in cuticle preparations (Fig. 2B,C).

We examined the dorsal closure process of *Djun* mutant embryos by staining with an anti-Engrailed (anti-En) antibody, which marks the border between the amnioserosa and the epidermis, and an anti-Spectrin antibody that stains the profiles of epidermal cells. In wild-type stage-14 embryos, the dorsal-most cells and most lateral epidermal cells have already elongated (Fig. 3A1), however, in *Djun* mutant embryos they remain polygonally shaped (Fig. 3A2) and dorsal closure never initiates. These defects can also be detected in embryos stained with anti-En (Fig. 3B). These results suggest that *Djun* is required for the initiation of dorsal closure.

The dorsal open phenotype may be attributable to incorrect patterning of the embryo at early stages, with dorsal epidermis defects appearing secondarily. To examine this possibility, we have used the expression pattern of Krüppel (Kr) as a marker for proper amnioserosa differentiation (Ray et al. 1991). We found that Kr is expressed in *Djun* mutant embryos in patterns indistinguishable from those observed in wild-type embryos (data not shown). The apparently normal development of *Djun* embryos up to stage 13 was also revealed by the normal expression of the segment polarity gene *en* (data not shown; Fig. 3B2). In conclusion, *Djun* is not required for embryonic segmentation.

Djun *regulates the DPP signal transduction pathway in the dorsal-most cells*

The absence of dorsal closure is the major defect associated with *Djun* mutant embryos. Because several members of the Dpp signal transduction pathway also exhibit this phenotype (Brummel et al. 1994; Nellen et al. 1994; Penton et al. 1994; Arora et al. 1995; Grieder et al. 1995; Letsou et al. 1995; Ruberte et al. 1995; Staehlong-Hampton et al. 1995), we tested whether *dpp* expression was affected in *Djun* mutant embryos. In wild-type embryos, *dpp* is expressed in a complex pattern that includes expression in the dorsal-most cells of the epidermis during germ-band retraction (St. Johnston and Gelbart 1987; Jackson and Hoffmann 1994; see Fig. 4A1,B1). We found that *dpp* transcripts are distributed normally in *Djun* mutants until the end of germ-band extension (data not shown). However, following the initiation of germ-band retraction, *dpp* expression at the dorsal-most cells is dramatically disrupted, whereas expression in the visceral

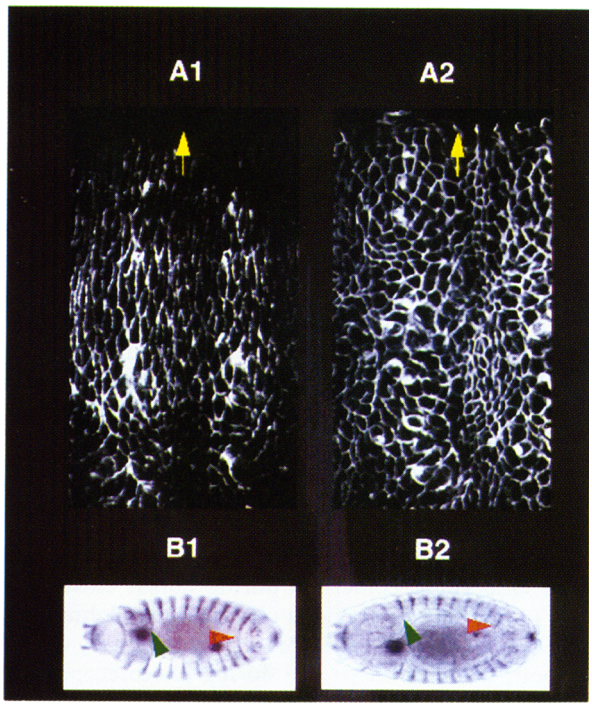

Figure 3. *Djun* mutation disrupts the initiation of dorsal closure. Epithelial cell shape changes during dorsal closure are revealed in wild-type (*A1,B1*) and *Djun* mutant (*A2,B2*) embryos. The cell profiles are illustrated with anti α-Spectrin antibodies (*A1,A2*). The border between amnioserosa and epidermis is marked with anti-En antibodies (*B1,B2*). In wild-type stage-14 embryos, epidermal cells in both the dorsal-most (arrow) and lateral positions have elongated (*A1*), but the dorsal-most cells (indicated by arrow) in *Djun* mutant embryo remains polygonal shape (*A2*). Whereas the anterior- and posterior-most En stripes moved dorsal-ward and joined together (see arrowheads in *B1,B2*) in wild-type stage-14 embryo (*B1*), they remain motionless in *Djun* mutant embryo (*B2*). Anterior is *left*. *A1* and *A2* are lateral views. *B1* and *B2* are dorsal views.

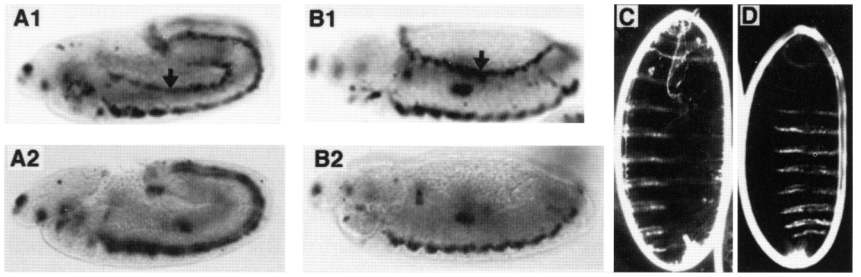

Figure 4. *Djun* regulates Dpp signal in the dorsal-most cells. *dpp* in situ hybridizations (*A1,A2,B1,B2*) to wild-type (*A1,B1*) or *Djun* mutant (*A2,B2*) embryos are shown. *dpp* expression is normal in *Djun* mutant embryos up to full germ-band extension (data not shown). During (*A2*, cf. with *A1*) and following (*B2*, cf. with *B1*) germ-band retraction, the *dpp* stripe of expression in the dorsal-most cells (arrows in *A1* and *B1*) is dramatically disrupted. (*C,D*) Ectopic expression of a constitutively active form of type I Dpp receptor TKV in the dorsal-most cells (see Materials and Methods for details) significantly rescues the dorsal open phenotype of homozygous $Djun^1$ embryos. Most embryos are rescued fully and develop normally as indicated by the completely retracted germ band and well-developed head structures (*C*). A small fraction of the embryos (~20%) display head defects (*D*) and variable dorsal defects probably reflecting variability in the level of expression of the activated TKV receptor.

mesoderm and the lateral ectoderm is normal (Fig. 4, cf. A2 with A1 and B2 with B1). These data suggest that DJUN specifically regulates, directly or indirectly, *dpp* expression at the dorsal-most cells of the epidermis.

To determine whether the Dpp signal transduction pathway acts downstream of DJUN in regulating dorsal closure, we expressed a constitutively form of the type I Dpp receptor TKV (TKV*) (Hoodless et al. 1996) in the dorsal-most cells of a *Djun* mutant embryo using the targeted ectopic expression system (Brand and Perrimon 1993). Two transgenes were introduced separately into a $Djun^1$ mutant background. One of them contains a cDNA-encoding TKV* cloned downstream of Gal-4-binding sites (Hoodless et al. 1996) and the other one expresses Gal4 under the control of the *pannier* (*pnr*) promoter (Calleja et al. 1996). Expression of TKV* rescues the dorsal open phenotype of *Djun* mutant embryos (Figs. 4C,D), consistent with the model showing that the function of DJUN during dorsal closure is to activate *dpp* expression in the dorsal-most cells of the embryonic epidermis.

Djun *regulates the organization of the leading edge*

Cell shape elongation along the dorsal–ventral axis is driven by the leading edge in the dorsal-most cells during germ-band retraction. The leading edge is similar to mammalian focal adhesion plaques. It is enriched with tyrosine-phosphorylated proteins, most notably paxillin, and also corresponds to the actin and myosin nucleation sites (Harden et al. 1996; Fig. 5). The movement of a molecular motor myosin with a filamentous actin substrate is believed to be driving cell shape changes (Young et al. 1993). We compared the distribution of phosphotyrosine, actin, and myosin in wild-type (Figs. 5A1,B1,C1) and *Djun* mutant embryos (Figs. 5A2,B2,C2). Although phosphotyrosine, actin, and myosin are gradually accumulated at the leading edge following dorsal closure progression (Fig. 5A1,B1,C1), this localization is disrupted totally in *Djun* mutant embryos (Fig. 5A2,B2,C2). These data suggest that DJUN regulates the initiation of dorsal closure through regulating the organization of the leading edge.

In the embryo, Djun *acts downstream of the JNK cascade but not downstream of Rl/MAPK*

Previous studies indicated that *Djun* is expressed maternally (Perkins et al. 1990; Zhang et al. 1990). To determine whether *Djun* is involved in earlier embryonic processes that may have been masked by the maternally derived product, we examined the effect of removal of the maternal contribution by examining the phenotype of embryos derived from females carrying homozygous *Djun* germ-line clones. Embryos lacking both maternal and zygotic *Djun* activity have similar phenotypes to those of homozygous mutant embryos (data not shown), indicating that removing maternal *Djun* activity has no or little effect on embryonic development. In particular, these embryos have no terminal defects, indicating that DJUN is not involved in the Torso RTK signaling pathway (Duffy and Perrimon 1994). In addition, these embryos, unlike zygotic DER (*Drosophila* EGF receptor) null mutant embryos (Ray and Schupbach 1996), differentiate cuticle with denticle belts and their segmenta-

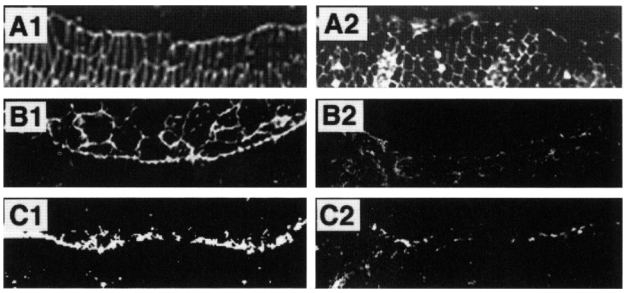

Figure 5. *Djun* controls the organization of the leading edge. The distribution of phosphotyrosine (*A1,A2*), filamentous actin (*B1,B2*), and nonmuscle myosin heavy chain (*C1,C2*) are shown in wild-type embryos (*A1,B1,C1*) and *Djun* mutant embryos (*A2,B2,C2*). Embryos were stained for filamentous actin with phalloidin or for phosphotyrosine and nonmuscle myosin heavy chain with antibodies. Whereas phosphotyrosine (*A1*), actin (*B1*), and myosin (*C1*) are accumulated along the leading edge in wild-type embryos, these accumulations are disrupted completely in *Djun* mutant embryos (*A2,B2,C2*). Anterior is to the *left*. All panels are lateral views.

tion appears normal (Fig. 2). These results suggest that *Djun* is not involved in RTK–RAS1–DRAF–Rl/MAPK signal transduction pathways during embryogenesis.

To test whether, and where, DJUN acts in the JNK signal pathway, we introduced constitutively activated *Djun* under control of the heat shock gene promoter and enhancer (*hs–Djun^{Asp}*) (Treier et al. 1995) into *DRac^{DN}*, *hep*, and *bsk* embryos (see introductory section). In addition, we also introduced *hs–Djun^{Asp}* into *Dsor1* (*Drosophila* Rl/MAPK) mutant embryos to determine whether activated Jun can rescue the defects associated with loss of RTK activities in the embryo. The heat shock treatment significantly rescued the dorsal open phenotype of *Drac^{DN}*, *hep*, and *bsk* embryos (Fig. 6) but had no effect on the *Dsor1* maternal effect phenotype.

In summary, *Djun* mutations exhibit dorsal closure defects that are also found in embryos missing *Drac*, *hep*, or *bsk* gene activities. They do not have any of the phenotypes expected if DJUN was a component of RTK pathways such as the Torso or DER signaling pathways. Furthermore, constitutively activated *Djun* rescues the mutant phenotype of components in the JNK pathway but not the mutant phenotype of a component in the Rl/MAPK pathway. These results suggest that DJUN is a specific target of JNK during embryogenesis but not of Rl/MAPK.

Djun *is not a target of Rl/MAPK in Sev signal transduction pathway for photoreceptor cell fate specification in the eye*

To characterize the function of *Djun* in Sev signaling, we examined the effects of *Djun* mutations on signaling by activated Sev (*sev^{S11}*) and activated Draf (*raf^{torY9}*) proteins expressed under control of the *sev* promoter and enhancer (Basler et al. 1991; Dickson et al. 1992) as well as its effect on an activated Rl/MAPK protein provided by the *rl^{Sem}* mutation (Brunner et al. 1994). Heterozygosity of *Djun* did not suppress the rough eye phenotype (Fig. 7; data not shown).

We also generated clones of cells homozygous for *Djun* following mitotic recombination in the eye to directly examine the role of DJUN in the eye. Clones of *Djun* mutant cells were similar in size and number to those found in the control (Fig. 7). These results indicate that *Djun*, like *bsk* (Riesgo-Escovar et al. 1996) but not like mutants in Sev RTK signal transduction pathway, is not required for photoreceptor cell differentiation in the eye.

Discussion

We have identified mutations in the *Drosophila* homolog of the mammalian proto-oncogene c-*jun* gene (*Djun*). In contrast to previous observations (Bohmann et al. 1994; Treier et al. 1995; Peverali et al. 1996), we find that both in the embryo and during photoreceptor cell determination, DJUN is not regulated by a pathway that involves MAPK. In the embryo, DJUN is a target of a pathway that involves JNKK/JNK. *Djun* mutations disrupt the initiation of dorsal closure suggesting that the JNKK/JNK/DJUN pathway is important for proper morphogenetic activity of cell sheets. One downstream target of DJUN is *dpp*. *dpp* transcription in the dorsal-most cells is disrupted in *Djun* mutants. Our data suggest that DJUN connects JNK signal transduction pathway to Dpp signal transduction pathway in regulating dorsal closure.

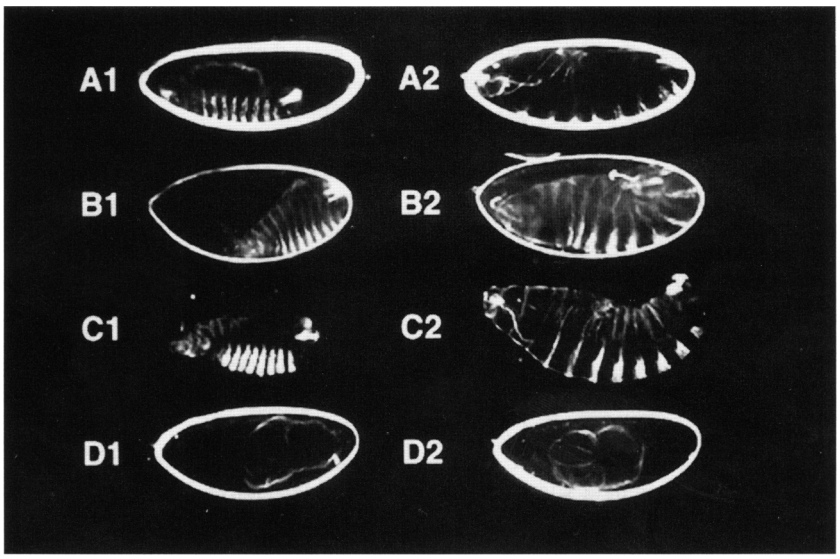

Figure 6. *Djun* is epistatic to *Drac*, *hep*, and *bsk*, but not to *Dsor1*. The dark-field cuticle pattern is of embryos of the following genotypes. (*A1*) *UAS–Drac$^{N17.1}$/hs–Gal-4*; (*A2*) *+/hs–Djun^{Asp}*; *UAS–Drac$^{N17.1}$/hs–Gal-4*; (*B1*) *hep^{1}*; (*B2*) *hep^{1}*; *+/hs–Djun^{Asp}*; (*C1*) *bsk^{1}/bsk^{1}*; (*C2*) *bsk^{1} hs–Djun^{Asp}/bsk^{1} hs–Djun^{Asp}*; (*D1*) *Dsor1^{LH110}* (derived from *Dsor1^{LH110}/Dsor1^{LH110}* germ-line clones; Lu et al. 1994); and (*D2*) *Dsor1^{LH110}*; *+/hs–Djun^{Asp}*. Embryos with one copy *hs–Djun^{Asp}* were heat-shocked for 30 min at 5–7 hr after egg laying. Embryos with two copies of *hs–Djun^{Asp}* are without heat shock treatment. Constitutively activated *Djun* significantly rescued the dorsal open phenotype of *Drac$^{N17.1}$*(*A2*), *hep^{1}* (*B2*), and *bsk^{1}*(*C2*), but does not rescue the mutant phenotype of *Dsor1^{LH110}*(*D2*). Embryos that lack both maternal and zygotic *Dsor1* function develop poorly differentiated cuticle that resemble the loss of DER signaling (Lu et al. 1994). This phenotype is not rescued by expression of activated Jun. In all panels anterior is *left*, and dorsal is *up*. *hs–Djun^{Asp}* is the constitutively activated form of *Djun* under heat shock promoter.

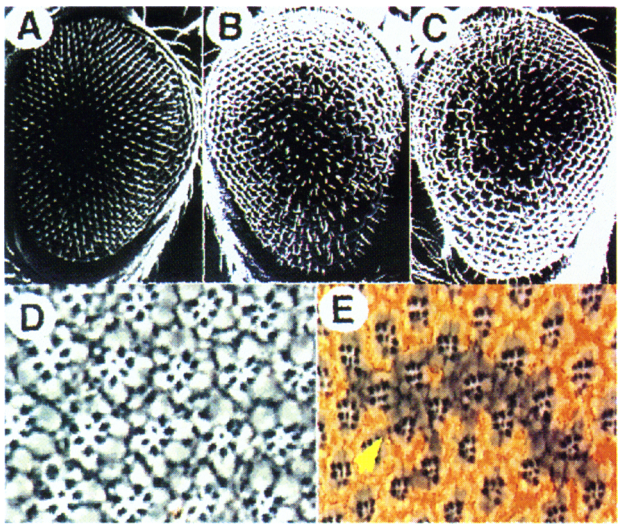

Figure 7. *Djun* is not required for Sev RTK signal transduction and specification of photoreceptor cell fates. (*A–C*) Scanning electron micrographs (SEMs) of adult eyes of the following genotypes. (*A*) Wild-type (Oregon R); (*B*) $raf^{torY9}/+$; (*C*) $raf^{torY9}/Djun^{1}$. Note that the raf^{torY9} rough eye phenotype is not suppressed by removal of one copy of $Djun^{1}$ (cf. *C* with *B*). $Djun^{1}$ also does not suppress the rough eye phenotypes of sev^{S11} and rl^{Sem} (data not shown). (*E,F*) Tangential sections of eyes of wild-type (*E*) and $Djun^{1}/+$ flies carrying a clone (arrowhead) of homozygous $Djun^{1}$ cells (marked by the absence of pigment granules). Note that all ommatidia in the mutant clone are wild type.

Djun *functions in the JNK pathway but not MAPK pathway in both the embryo and in the eye*

Several lines of evidence suggest that DJUN functions in the JNKK/JNK pathway but not in RTK/MAPK signaling pathways in the embryo. First, embryos that lack both maternal and zygotic *Djun* activity have similar dorsal open phenotypes. The absence of additional phenotypes, such as terminal defects or poorly differentiated cuticle in germ-line clone-derived embryos, indicates that DJUN is not a component of either the Torso or DER/EGFR RTK signaling pathways. Second, the constitutively activated form of *Djun* rescues the mutant phenotypes of members of the JNK pathway but not of *Dsor1*, a member of the Rl/MAPK pathway. These observations place DJUN downstream of JNK but not of Rl/MAPK.

In addition, there are two lines of evidence against a function of DJUN in the Rl/MAPK pathway in the eye. First, homozygous *Djun* clones induced in the developing *Drosophila* eye imaginal disc do not affect photoreceptor differentiation. Second, reduction of *Djun* activity by half does not suppress ectopic photoreceptor cell differentiation triggered by constitutively activated forms of either the Sev, Raf, or Rl/MAPK. These genetic results on the function of *Djun* in the eye are consistent with the observation that *bsk/JNK* also does not affect photoreceptor cells differentiation (Riesgo-Escovar et al. 1996).

Our conclusion is in contrast to the previous observations (Bohmann et al. 1994; Treier et al. 1995; Peverali et al. 1996), which reported that DJUN was a target of Rl/MAPK in Sev signaling. A simple explanation is that the previous experiments were performed under nonphysiological conditions. The dominant negative and constitutively activated forms of DJUN employed in these experiments may bind to factors regulated by the MAPK pathway and block their normal functions, or the modified Jun proteins may compete with factors in the MAPK pathway for binding to the promoter elements of downstream target genes. Alternatively, there may be another *jun* gene in the *Drosophila* genome.

Role of the JNK/DJUN pathway during dorsal closure

In embryos, DJUN is a target of a pathway that involves JNKK/JNK. *Djun* mutations disrupt the initiation of dorsal closure, suggesting that it is important for proper morphogenetic activity of cell sheets. DJUN may accomplish its function in this process by controlling Dpp signal transduction pathway in the leading edge. Therefore, this is a relay process of four members: JNK pathway to DJUN, DJUN to *dpp*, and Dpp pathway to the leading edge, which drives the cell shape change and cell movement. A model for the relay signal transduction pathway is presented in Figure 8.

The requirement of a Drac–Hep–Bsk–DJUN signal transduction pathway for dorsal closure during embryogenesis is supported by the following data. First, mutations in *Djun* (Fig. 2), *bsk*, *hep*, and expression of dominant-negative forms of *Drac* ($Drac^{DN}$) (Luo et al. 1994; Glise et al. 1995; Harden et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996) disrupt dorsal closure; second, activated DJUN rescues the dorsal open phenotype of $Drac^{DN}$, *hep*, and *bsk* embryos; third, Hep phosphorylates and activates Bsk, and Bsk phosphorylates DJUN in vitro (Sluss et al. 1996); fourth, mammalian JNK is activated by Rac-family GTPases (Coso et al. 1995; Hill et al. 1995; Minden et al. 1995).

One downstream target of DJUN is *dpp*. *dpp* transcription in the dorsal-most cells is disrupted by *Djun* mutations. Targeted expression of constitutively activated forms of the type I Dpp receptor TKV in the dorsal-most cells rescues the dorsal open phenotype of *Djun* mutations. Mutations in components of the Dpp signaling pathway exhibit dorsal open phenotypes (see introductory section), indicating that the Dpp signaling pathway is required for dorsal closure. These data suggest that the role of DJUN during this process is to relay the JNK pathway to the Dpp pathway through specifically regulating *dpp* expression in the dorsal-most cells.

Members of the mammalian Jun, Fos family belong to the AP-1 transcription factor family. All members of this group share the same binding specificity for a palindromic DNA sequence, the AP-1-binding site, which functions as a signal-responsive transcription control element in a number of cellular and viral promoters (Angel et al. 1987; Bohmann et al. 1988). The DNA-binding competent and transcriptional active form of AP-1 is a

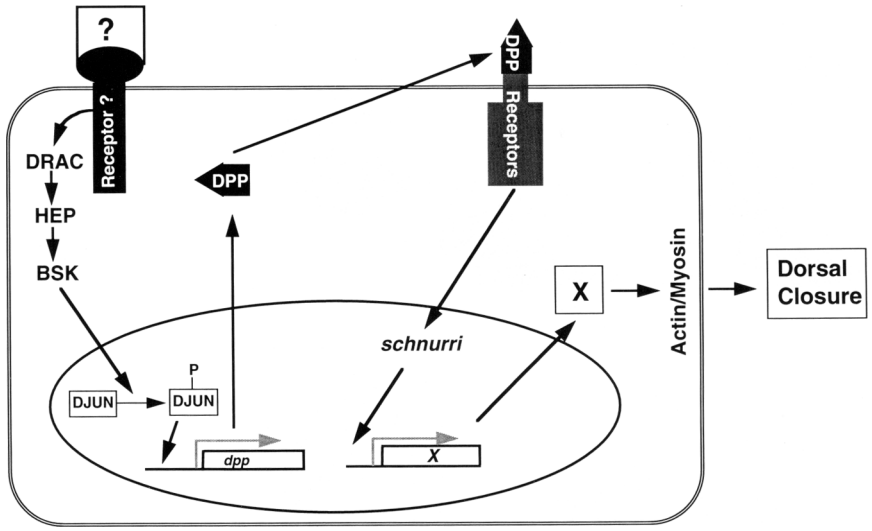

Figure 8. Model of the role of DJUN in dorsal closure formation. See text.

protein dimer formed by two family members through a coiled–coil interaction. *Djun* is expressed ubiquitously in embryos (Perkins et al. 1990; Zhang et al. 1990), it may cooperate with another locally expressed factor to turn on *dpp* expression in the dorsal-most cells. It is possible that DFOS represents such a partner of DJUN in regulating *dpp* as *Dfos* is expressed in the dorsal ectoderm and amnioserosa at 8 hr of embryogenesis (Perkins et al. 1990; Rusch and Levine 1997). However, it is equally likely that an unknown transcription factor interacts with DJUN in regulating *dpp* expression in the dorsal-most cells.

Dorsal closure formation: a model

In Figure 8 we propose a model for the process of dorsal closure formation. DJUN regulates *dpp* expression in the dorsal-most cells and Dpp signal is also transduced in these cells, this relay process may be carried out in the same cells. The JNK pathway first phosphorylates and activates DJUN, DJUN then regulates *dpp* expression in the dorsal-most cells. Subsequently, the Dpp protein is secreted, binds to its receptors on the cell surface, and signals back to the nucleus to regulate gene *X* expression, whose product organizes the leading edge to drive cell shape changes for dorsal closure. It should be noted that our experiments did not address whether DJUN acts directly on the *dpp* promoter or not, such that it is possible that the effect of DJUN on *dpp* expression is indirect.

The nature of the signal/receptor that activates the JNK/DJUN pathway in the dorsal epidermis remains to be characterized. Recent studies in mammalian cells have shown that the stress-activated protein kinases (SAPKs)/JNKs can be activated by inflammatory cytokines such as tumor necrosis factor α (TNFα) and IL-1, as well as G protein-coupled receptors (Coso et al. 1995; Davis 1995; Hill et al. 1995; Minden et al. 1995). The *Drosophila* JNK/DJUN may be activated by similar signals.

Concluding remarks

An emerging concept of pattern formation is the relay of signal transduction pathways to pattern tissues. For examples, Dpp expression is regulated by the Hedgehog signaling pathway during anteroposterior patterning of the wing disc (Lawrence and Struhl 1996). Similarly, localized activation of the Notch signaling pathway defines the late domain of expression of Wingless during dorsoventral patterning of the wing disc (Kim et al. 1996). Here we have demonstrated another such example whereby the JNK/DJUN pathway regulates dorsal closure by regulating Dpp expression.

Materials and methods

Drosophila *stocks*

The screen for specific mutations in the 46C–F regions was described in Bour et al. (1995). $Djun^{1}$ is among these mutations and was originally named *76–19*. *l(2)IA109* and bsk^{1} were obtained from the Tübingen Stock Collection; hep^{1} from S. Noselli (University of Toulouse, France); UAS–TKV* [described as UAS–TKV(Q199D) in Hoodless et al. 1996] from M. O'Connor (University of California, Irvine) via S. Newfeld (Harvard University, Boston, MA); and *pnr*–Gal-4 from the Bloomington Stock Center. $Dsor1^{LH110}$ is described in Lu et al. (1994). $Drac^{N17.1}$ transgenic flies were from L. Luo (University of California, San Francisco) and heat shock *Djun* cDNA transgenic flies from M. Mlodzik (European Molecular Biology Laboratory, Heidelberg, Germany). *hs-Gal-4* was generated by A. Brand in this laboratory. To test for genetic interactions between *Djun* and components of the Sev pathway, the following stocks were obtained from E. Hafen (University of Zürich, Switzerland): $sev^{S11.1}$ (Basler et al. 1991), $sE\text{–}raf^{torY9}$ (Dickson et al. 1992), and rl^{Sem} (Brunner et al. 1994).

Flies were raised on standard *Drosophila* media at 25°C, un-

less indicated. Chromosomes and mutations that are not described in the text can be found in Lindsley and Zimm (1990).

Genetic rescue

A P-element rescue plasmid was constructed by inserting a 5.2-kb *Xho*I–*Xba*I fragment containing *Djun* into the pw8 vector (E.S. Goldstein, unpubl.). The insert contained 2.2 kb upstream and ~0.8 kb of downstream sequence. Germ-line transformants were produced by standard methods (Spradling and Rubin 1982) following injection of the rescue and a helper plasmid. Four independent X-chromosome lines were obtained and crossed in *Df(2R)X1/CyO*, a deficiency that uncovers the 46C–F region. These lines were then crossed to zygotic lethal mutations and scored for the presence of straight wing flies.

Molecular biology techniques

Genomic DNAs were isolated from adult flies, digested with restriction enzymes, and analyzed on Southern blots using *Djun* cDNA as a probe under standard conditions (Sambrook et al. 1989).

PCR primers 5′-GGAATTCCCTTCATCCGAATCAGATTGACG-3′ and 5′-GGAATTCCTCATCATTCTCCCCGGCT-3′ were used to amplify genomic DNA fragments from $Djun^2$ homozygous mutant embryos, and the product was subcloned. DNAs from five separate clones were sequenced and all identified the same AAG to TAG mutation at amino acid 72.

Antibody staining and immunohistochemistry

Antibody and phalloidin stainings and confocal microscopy of embryos were performed as described previously (Harden et al. 1995). Embryos aged 10–14 hr at 25°C were used to study the process of dorsal closure. Anti-α-Spectrin antibody (obtained from D. Branton, Harvard University, Boston, MA) were used at 1:1000 dilution, anti-nonmuscle myosin antibody (obtained from D. Kiehart, Duke University Medical School, Durham, NC) at a 1:500, and anti-Engrailed antibody (obtained from R. Holmgren, Northwestern University, Evanston, IL) at 1:200.

In situ hybridization of whole-mount embryos using digoxigenin-labeled antisense of a *dpp*-coding DNA probe was performed as described (Hou et al. 1995).

Genetic interactions

To test for interactions between *Djun* and $Drac^{DN}$, *hep*, *bsk*, and *Dsor1*, the following stocks were constructed: $hep^1/FM7$; $hs\text{–}Djun^{Asp}/+$ and $FM7/Y$; $hs\text{–}Djun^{Asp}/+$, $bsk\ hs\text{–}Djun^{Asp}/Cyo$, $hs\text{–}Djun^{Asp}/+$;$UAS\text{–}Drac^{N17.1}/UAS\text{–}Drac^{N17.1}$, and $hs\text{–}Djun^{Asp}/+$; *hs–Gal-4/hs–Gal-4*. Embryos with genotypes hep^1/Y; $hs\text{–}Djun^{Asp}/+$, $bsk\ hs\text{–}Djun^{Asp}/bsk\ hs\text{–}Djun^{Asp}$, $hs\text{–}Djun^{Asp}/+$; $UAS\text{–}Drac^{DN}/hs\text{–}Gal\text{-}4$ were collected for 2 hr and aged to 5–7 hr after egg laying before a 30–60 min heat shock. Following heat shock, the embryos were aged for another 24 hr at 25°C before cuticle preparation. The cuticle phenotypes were compared for embryos that are either with and/or without the constitutively activated *Djun* transgene.

Targeted ectopic expression of an activated form of type I DPP receptor TKV, TKV*, to the dorsal-most cells was achieved using the Gal-4/UAS system (Brand and Perrimon 1993). A UAS–TKV* element on the third chromosome (Hoodless et al. 1996) was induced in $Djun^1$ homozygous embryos. The inducer line was the *pnr*–Gal-4 line on the third chromosome (Calleja et al. 1996).

Clonal analysis and histology

Germ-line clones of *Djun* were generated using the "FLP–DFS" technique (Chou and Perrimon 1996).

For generating clones in adult eyes, the FLP–FRT recombinase system (Xu and Rubin 1993) was used. Second chromosomes carrying *Djun* mutations and FRT^{42D} were constructed. Virgin females of genotype FRT^{42D} w^+FLP were mated with males of genotype w; FRT^{42D}–*Djun/CyO*. Clones were induced in first instar larvae and examined in adult eyes.

Scanning electron microscopy and histological sections of eyes were done as described (Basler et al. 1991).

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