**Drosophila** Jun kinase regulates expression of *decapentaplegic* via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure

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During *Drosophila* embryogenesis, ectodermal cells of the lateral epithelium stretch in a coordinated fashion to internalize the amnioserosa cells and close the embryo dorsally. This process, dorsal closure, requires two signaling pathways: the *Drosophila* Jun-amo-no-terminal kinase (DJNK) pathway and the Dpp pathway. We have identified mutations in *DJun* and show that DJNK controls dorsal closure by activating DJun and inactivating the ETS repressor Aop/Yan by phosphorylation. DJun and Aop regulate *dpp* expression in the most dorsal row of cells. Secreted Dpp then instructs more ventrally located cells to stretch. Our results provide a causal link between the DJNK and Dpp pathways during dorsal closure. Interestingly, in vertebrates, transforming growth factor-β and c-Jun regulate collagenase gene expression during wound healing, a process that also involves the closing of an epithelial sheath.

[Key Words: Jun kinase; TGF-β; ETS-domain protein; Jun; signaling; development]

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During the development of multicellular organisms, extracellular signals regulate cell division, cell fate, and changes in cell shape. It is important to understand how the large number of signals elicit selectively and reproducibly the correct cellular responses. The availability of genetically tractable organisms in which these processes can be studied in vivo has been instrumental in the elucidation of various signaling pathways (Engelberg et al. 1994; Zipursky and Rubin 1994; Artavanis-Tsakonas et al. 1995; Kayne and Sternberg 1995; Kenyon 1995; Wassarman et al. 1995; Domínguez and Hafen 1996). The results obtained can be broadly applied, because the molecules required in these processes have proven to be conserved between species. The process of dorsal closure during *Drosophila* embryogenesis is a particularly well-suited model to study not only individual signaling pathways, but also the interplay between such pathways, because genetic evidence indicates that the closure process is dependent on both Jun kinase and transforming growth factor-β [TGF-β] pathways (Affolter et al. 1994; Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996).

Dorsal closure is defined as the coordinated changes in cell shape in the lateral epithelium that occur at midembryogenesis in *Drosophila melanogaster* (Campos-Ortega and Hartenstein 1985). Lateral epithelial cells elongate and narrow to cover the dorsal half of the embryo, such that the stretching epithelia meet at the dorsal midline. Previous studies have shown that the onset of dorsal closure can be visualized by actin and myosin accumulation beneath the apical membranes of the most dorsal row of cells of the lateral epithelium, the leading edge cells (Young et al. 1991, 1993). These cells elongate or stretch dorsally over adjacent amnioserosa cells. Subsequently, the ectodermal cells located ventral to the leading edge also stretch until the cells meet at the dorsal midline. Here, the leading edge cells interdigitate to close the ectoderm (Ring and Martinez-Arias 1993, Young et al. 1993).

A large number of embryonic lethal mutations block the process of dorsal closure producing a characteristic dorsal open phenotype. The gene products identified by these mutations can be grouped into two categories of proteins: [1] cytoskeletal and extracellular matrix components (Wieschaus et al. 1984; Wilcox et al. 1989; Young et al. 1993; Fehon et al. 1994; Miyamoto et al. 1995; Borchelli et al. 1996), and [2] signaling components of the DJNK and Dpp pathways (Jürgens et al. 1984; Nüsslein-Volhard et al. 1984; Affolter et al. 1994; Brummel et al. 1994; Penton et al. 1994; Terracol and...
Lengyl 1994; Glise et al. 1995; Grieder et al. 1995; Harden et al. 1995; Ruberte et al. 1995; Nellen et al. 1996; Riesgo-Escovar et al. 1996; Sluss et al. 1996).

So far, two components of the DJNK pathway have been shown to be required for dorsal closure: Drosophila Jun amino [N]-terminal kinase kinase (DINNK) encoded by hemipterous (hep) [Glise et al. 1995] and Drosophila Jun amino [N]-terminal kinase (DJNK) encoded by bas- ket (bsk) [Riesgo-Escovar et al. 1996, Sluss et al. 1996]. In the absence of DINKK or DJNK function, lateral epithelial cells fail to stretch. A similar phenotype is observed in dorsal closure has been precluded by the fact that

tations in (put), and Gelbart 1987; Padgett et al. 1987; St. Johnson and Hoffmann 1994). However, we note that dpp is expressed in the leading edge cells during dorsal closure (St. Johnson and Gelbart 1987).

Here we provide evidence for a functional link between the DJNK and Dpp pathways by showing that the DJNK pathway controls dorsal closure at least in part by regulating dpp expression in the leading edge cells. We show that dpp expression is reduced or absent in leading edge cells of embryos lacking DJNK function. We iden-
tify two nuclear targets of the DJNK pathway that are required for dorsal closure, the transcription factors DJun and the ETS domain repressor Aop/Yan. Mutations in DJun block dorsal closure and reduce dpp expression in the leading edge cells. Conversely, mutations in the ETS domain repressor Aop/Yan lead to dpp overexpression. Expression of an activated Dpp receptor, Tkv-QD, in the lateral epithelium partially restores dorsal closure in the absence of DJNK function. These findings suggest that the DJNK pathway governs dorsal closure at least partially by regulating dpp expression via phosphorylation of DJun and Aop.

Results

DJun mutation affects dorsal closure

Given the fact that in vertebrate cells, Jun kinase acti-
vates the transcription factor Jun [Derijard et al. 1994; Kallunki et al. 1994], we wondered whether DJNK controls dorsal closure by activation of DJun. Therefore, we searched for embryonic lethal mutations with a dorsal open phenotype in the 46D-E region where DJun has been localized [Perkins et al. 1990; Zhang et al. 1990]. l(2R)IA109 maps to the DJun region and embryos with this mutation are phenotypically very similar to bsk mutant embryos lacking DJNK [Fig. 1C; Nüsslein-Volhard et al. 1984]. There is no difference in the strength of the phenotype of embryos homozygous for l(2R)IA109 or heterozygous over a deficiency that uncovers the locus suggesting that l(2R)IA109 is a complete loss-of-function mutation. As in bsk mutant embryos, the dorsal open phenotype in this mutant is caused by failure of the lateral epithelial cells to stretch (Fig. 1F). The evidence that l(2R)IA109 is a complete loss of function allele of DJun is twofold: Rescue experiments involving the repeated induction of a heat inducible DJun transgene [Bohmann et al. 1994] rescued homozygous l(2R)IA109 embryos to pharate adults. Sequencing of the DJun coding region from the l(2R)IA109 mutant revealed a single base change at position 651 of the DJun sequence that introduces a stop codon. Therefore, l(2R)IA109 encodes a truncated DJun protein lacking the bZip DNA-binding and dimerization domain and the amino-terminal phospho-
sylation sites [Fig. 2]. DJun function does not appear to be essential for earlier processes during embryogenesis, because embryos lacking both maternal and zygotic DJun product possess a dorsal open phenotype identical to that of embryos lacking only zygotic DJun function [Fig. 1G].

Genetic evidence that DJun is a critical target in the DJNK pathway during dorsal closure

The similarity of the DJNK (Bsk) and the DJun l(2R)IA109] mutant phenotypes suggests that DJNK controls dorsal closure at least in part by phosphoryla-
tion of DJun. This idea is supported by genetic evidence: The partial dorsal open phenotype of weak bsk mutations is strongly enhanced by removing a wild-type copy of DJun or by overexpressing a dominant negative form of DJun [DJunDZarp, [Bohmann et al. 1994]] [Fig. 1H,I]. Overexpression of either wild-type DJun or an activated form of vertebrate Jun [Treier et al. 1995] in which the JNK phosphorylation sites have been replaced by acidic residues suppresses the dorsal open phenotype of bsk mutants [Fig. 1K]. These data indicate that elevated Jun activity can substitute for DJNK function during dorsal closure, supporting the notion that DJNK controls dorsal closure by increasing DJun activity.

DJun is not required for cell fate determination in the developing eye

The similarities of mutant DJun and Bsk embryonic phe-
notypes indicate that the two genes are required in the same developmental pathway during embryogenesis. However, different requirements for DJun and DJNK during postembryonic development have been reported. Based on mutant eye phenotypes caused by overexpression of dominant negative DJun and activated mammalian c-Jun, it has been proposed that DJun is required for the specification of cell fate in the developing eye [Boh-

mann et al. 1994; Treier et al. 1995; Peverali et al. 1996]. On the other hand, we have shown recently that loss of DJNK function in clones of bsk mutant cells does not interfere with photoreceptor cell specification [Riesgo-Escovar et al. 1996]. Here we have directly tested the requirement for DJun function in the eye by clonal analysis of the newly identified DJun loss-of-function
or zygotic DJun function. The phenotype is indistinguishable from a DJun zygotic null mutation [C]. [H,F] DJun mutant or expression of dominant negative DJun [DJun\$z21b$] enhances the bsk mutant phenotype. [I] Cuticle of a DJun bsk embryo. Df(2L)flp147E represents a complete loss-of-function mutation for bsk. Whereas the dorsal open phenotype of this mutant is somewhat stronger than that shown for bsk1 [B], these Df(2L)flp147E embryos still close on the posterior dorsal side. Removal of one copy of DJun enhances this phenotype such that the entire dorsal side is open. [J] Cuticle of a DJun bsk embryo that was heat shocked for one hr at 37°C during development. Note that the dorsal aspect of the cuticle is completely open. [K] Overexpression of DJun and Jun\$act$ suppress the bsk phenotype. [L] Cuticle of a DJun bsk embryo heat shocked for 1 hr at 37°C during the first 10 hr of embryonic development. The cuticle is completely closed. (C) An embryo with no maternal or zygotic DJun function. The phenotype is indistinguishable from a DJun zygotic null mutation [C]. [I,F] DJun mutant or expression of dominant negative DJun [DJun\$z21b$] enhances the bsk mutant phenotype. While loss of aop function in the eye results in the differentiation of multiple R7 photoreceptor cells, overexpression of a mutant Aop protein (Aop\$act$) which cannot be phosphorylated by MAP kinase, blocks R7 development [Rebay and Rubin 1995]. The suppression of the bsk phenotype by aop suggests that the Aop protein also

**The ETS repressor Aop/Yan is required for dorsal closure**

Amongst the relatively large number of known mutants with defects in dorsal closure we found that aop acts as a strong suppressor of the bsk phenotype [Fig. 4B,D]. aop encodes an ETS repressor protein also known as Yan [Lai and Rubin 1992; Tei et al. 1992; Rogge et al. 1995]. The phenotype of aop loss of function mutations is pleiotropic [Rogge et al. 1995]. In the lateral epidermis, the leading edge cells stretch but other cells are irregularly arranged and some are still dividing [Fig. 4E-H]. Aop is a target of the MAP kinase encoded by the rolled (rl) gene in the developing eye and is involved in the specification of photoreceptor cell fate [Brunner et al. 1994; O’Neill et al. 1994]. While loss of aop function in the eye results in the differentiation of multiple R7 photoreceptor cells, overexpression of a mutant Aop protein (Aop\$act$) which cannot be phosphorylated by MAP kinase, blocks R7 development [Rebay and Rubin 1995]. The suppression of the bsk phenotype by aop suggests that the Aop protein also
functions as a repressor of dorsal closure. Therefore, we tested whether Aopact interferes with dorsal closure by expressing it in the lateral epidermis during embryogenesis. Overexpression of Aopact in the cells of the lateral epithelium prevented dorsal closure and resulted in dead embryos with a strong dorsal open phenotype (Fig. 3J). The genetic interaction and the fact that Aop is phosphorylated by DJNK in vitro (Fig. 5) suggest that DJNK controls dorsal closure by regulating the activity of the two antagonistic transcription factors: DJun and Aop. Phosphorylation of DJun results in its activation whereas phosphorylation of Aop results in its inactivation.

Expression of dpp in the leading edge cells is regulated by DJun activity

Thus far, we have established that DJun activity and probably also the ETS repressor Aop are regulated by DJNK during dorsal closure. What are the target genes whose expression is regulated by these transcription factors? Two genes, puckered (puc) and dpp, are expressed in the leading edge cells during dorsal closure. puc encodes a CI-100-like MAP kinase phosphatase (A. Gambel, E. Martin-Blanco, and A. Martinez Arias, pers. comm.) and its expression is dependent of DJNK and DJNKK function [Glise et al. 1995; Riesgo-Escovar et al. 1996]. Whereas expression of puc at this stage is restricted to the leading edge cells, dpp is expressed in a highly dynamic pattern in other cells as well (St. Johnson and Gelbart 1987; Schwyter et al. 1995). To test whether dpp expression is also controlled by DJNK activity, we examined its expression in embryos with reduced maternal and zygotic bsk function. From 48 embryos undergoing dorsal closure that were derived from females with homozygous bsk mutant germ cells and males heterozygous for a deficiency of bsk, 26 embryos had no, or a strongly reduced, expression of dpp in the leading edge cells. The rest of the dpp expression pattern was not affected and, therefore, served as an internal control (Fig. 6C,D). The frequency of embryos with reduced or absent dpp expression in the leading edge cells correlates well with the fraction of embryos [50%] that received the bsk deficiency from the male and, hence, display a bsk mutant phenotype. This indicates that the late expression of dpp in the leading edge cells depends on the activation of the JNK signaling pathway.

As anticipated, dpp expression in the leading edge cells is also dependent on DJun and aop function: dpp expression is reduced, or absent, in DJun [Fig. 6G] and in embryos expressing aopact (Fig. 6H,I). Conversely, dpp expression is expanded in aop mutant embryos [Fig. 6J] and in embryos overexpressing either the activated mammalian c-Jun or wild-type DJun [Fig. 6K,L]. The DJun-induced expansion of dpp expression is suppressed by a deficiency of bsk [Fig. 6M] suggesting that the ectopic DJun function is also dependent on DJNKK activity. Whereas expression of DJunbZIP in wild-type embryos does not reduce dpp expression in the leading edge cells significantly, it blocks dpp expression in embryos lacking zygotic DJNKK function [Fig. 6N,O]. We note that loss of DJun function affects dpp expression only in the leading edge cells but not, for example, in the visceral mesoderm. In contrast, ubiquitous expression of Junact results in an expansion of dpp expression not only in the lateral epidermis, but also in the visceral mesoderm, supporting the idea that results obtained from gain-of-function mutations such as hs-Junact may not reflect the normal function of the corresponding gene.

The Dpp signaling pathway is required for dorsal closure

Given the expression of dpp in the leading edge cells and the dependence of this expression on the activity of the DJNK pathway, we wondered whether Dpp signaling is required for the process of dorsal closure. Owing to the early requirement of dpp function in specification of dor-
sal-ventral cell fates and the lack of specific mutations that block only the late dpp expression, the analysis of loss-of-function mutations in dpp does not indicate whether dpp function is also required for the late process of dorsal closure. Dorsal open phenotypes have been reported, however, for loss-of-function mutations in the genes coding for two Dpp receptors, tkv and put (Fig. 7A; Affolter et al. 1994; Brummel et al. 1994; Nellen et al. 1994; Penton et al. 1994). Embryos mutant for tkv or put display a milder, but nevertheless similar, phenotype to that observed in bsk mutant embryos (Fig. 7A,C). The mutant embryos exhibit a dorsal hole in the central portion of the embryo. In contrast to bsk mutants, the dorsal anterior and posterior ends are closed normally and appear to be bent inwards (Fig. 7A; Affolter et al. 1994; Brummel et al. 1994; Nellen et al. 1994; Penton et al. 1994).

To examine the cell shape changes during dorsal closure in tkv and put mutant embryos, the mutant embryos were stained with the anti-Coracle antiserum (Fe-hon et al. 1994). Whereas the leading edge cells of tkv and put mutant embryos are extremely elongated and stretched, all other cells of the lateral epidermis are round like the cells in bsk mutant embryos (Fig. 7B). Therefore, in the absence of either of these two receptors for Dpp, the leading edge cells are still able to undergo a change in cell shape. Adjacent, more ventral epithelial cells, however, fail to stretch. Because the expression of dpp in the leading edge cells is controlled by the activity of the DJNK pathway and block of the reception of the Dpp signal in tkv and put mutants results in a failure of the ventral cells to change shape, we suggest that Dpp is involved directly or indirectly in the induction of the cell shape changes in lateral epidermal cells ventral to the leading edge.

To test whether activation of the Dpp signaling pathway is sufficient to induce dorsal closure in the absence of DJNK signaling, we induced ubiquitous ectodermal expression of transgenes encoding dpp or tkvQD (Nellen et al. 1996), an activated form of the Dpp receptor Tkv. Ectodermal expression of either dpp or tkvQD in a wild-type background results in dorsalized embryos that undergo normal dorsal closure [Fig. 7D,G]. Similarly, the cuticle of bsk embryos was partially dorsalized by the expression of either dpp or tkvQD, but the dorsal hole characteristic of bsk mutant embryos was much reduced in size (Fig. 7E,H,I). Cora staining of bsk mutant embryos ectopically expressing dpp in the ectoderm suggest that lateral ectodermal cells underneath the leading edge cells also stretch [data not shown]. Hence, expression of dpp or tkvQD in bsk mutant embryos resulted in a partial rescue of the dorsal open phenotype. Thus, activation of

Figure 4. The ETS domain repressor Aop is required for dorsal closure. (A–D) aop acts as a dominant suppressor of bsk. (A) Df(2L)flp147E mutant embryo. (B) aopQD,Df(2L)flp147E/+; Df(2L)flp147E mutant embryo. This embryo has only a small dorsal anterior hole. (C) bsk1/Df(2L)flp147E embryo derived from a bsk1 female germ line clone. Removal of maternal and zygotic bsk function results in a complete dorsal-open phenotype. (D) aopp, bsk1/QD, Df(2L)flp147E mutant embryo derived from an aopQD, bsk1 female germ line. Note that the reduction of aop function during oogenesis results in a marked suppression of the dorsal-open phenotype leaving only a small anterior dorsal hole [white arrow]. (E–H) aop mutations show pleiotropic effects in the lateral epithelium during dorsal closure. (E) Cuticle of a aopQD homozygous mutant embryo. The dorsal cuticle lacks dorsal hairs and has a convoluted appearance. (F) Anti-Cora stainings of the lateral epithelium of aopp embryos during dorsal closure. Cells stretch, although they appear more disorganized and are variable in size. The white arrow marks the leading edge cells. (G,H) wild-type [G] and aopQD [H] stage 13 embryos stained with anti-Tubulin. Note the ectopic foci of dividing cells [black arrow] in the aopQD mutant epithelium. (I) Expression of aopp phenocopies the bsk mutant phenotype: (I) Ventral view of the cuticle of a Df(2L)flp147E homozygous embryo, the arrow marks the Filzk6rper. (J) Ventral view of a UAS-AopQD;69B Gal4 embryo. Except for the rudimentary mouth hooks and Filzk6rper [arrow], the cuticular phenotype closely resembles that of bsk mutants shown in I.
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Figure 5. JNK phosphorylates Aop. In vitro kinase assay of JNK with AopA as substrate (Rebay and Rubin 1995). (Lane 1) AopA-GST; (lane 2) GST alone. JNK was immunoprecipitated from larval cell-free extracts and used for the kinase reactions followed by SDS page and autoradiography. Control experiments in which Mapk and JNK were immunoprecipitated from aliquots of the same larval extracts and used for in vitro kinase assays of bacterially produced AopA showed that both were able to phosphorylate the AopA-GST fusion protein (data not shown).

Figure 6. dpp expression in the leading edge cells is regulated by bsk, aop, and DJun. All panels show embryo whole mounts that had been in situ hybridized. For all panels except E the probe was an antisense dpp probe; for E it was an antisense tkv probe that is shown for comparison. (A,F) dpp phase III expression in wild-type embryos (Oregon R) at stage 11 and 13, respectively. dpp expression in the leading edge cells (arrow in F) is maintained from germ band extension to the end of dorsal closure. (B–D) dpp expression in bsk mutant backgrounds. (C) bsk1/+ embryo derived from a bsk1 germ line. This embryo is phenotypically wild-type and shows normal dpp expression. (C,D) bsk1/Df(2L)F1p147E embryo derived from a bsk1 germ line. These embryos would develop a strong dorsal-open phenotype and show reduced [C] or absent [arrow in D] dpp expression. (E) A wild-type embryo hybridized with a tkv antisense probe. (G) A homozygous DJunA10X embryo. The arrow marks reduced dpp expression in the leading edge cells. (H,I) UAS-AopAc1; 69B-Gal4 embryos. Note the reduced dpp expression in the leading edge cells caused by the expression of AopAc1. Conversely, in J, an aop1 mutant embryo has ectopic dpp expression [arrow] in rows of cells underneat the leading edge cells. (K,L) Ectopic expression of JunAc1 or DJun induces ectopic dpp expression. Embryos in K–O were heat shocked for 1 hr, left 3–4 hr to recover, and fixed subsequently. (K) dpp phase III expression domains are expanded in a hs-JunAc1 embryo. (L) Strong dpp expression in hs-DJun embryos. (M) Df(2L)F1p147E. hs-DJun embryo. dpp expression is reduced [compare with L] in the presence of strongly reduced JNK activity. (N) hs-DJunb2Zip and (O) Df(2L)F1p147E/+; hs-DJunb2Zip embryo. Expression of Junb2Zip results in reduced dpp expression only in the presence of reduced JNK function (N,O). (A,B) Dorsolateral views; (C,D) dorsal views; (E,O) are lateral views.
DJNK pathway controls dpp expression via Aop and DJun

The similarities between the embryonic phenotypes of DJun and bsk mutants support the idea that the functions of DJun and DJNK are closely linked during embryogenesis. Although it was shown previously that DJNKK and DJNK functions are required for the expression of puc in the leading edge cells (Glise et al. 1995; Riesgo-Escovar et al. 1996), it was not clear whether the DJNK pathway controls dorsal closure via the transcriptional regulation of target genes or whether it also acts more directly by phosphorylating components of the cytoskeleton. Because loss of DJun, which we and others have shown to be phosphorylated by DJNK in vitro (Riesgo-Escovar et al. 1996; Sluss et al. 1996), prevents changes in cell shape in much the same way as loss of DJNK does, it appears that DJNK controls the cell shape change by the regulation of target genes via DJun.

Although our data suggest that DJun is not essential for cell fate specification in the eye, another transcription factor, the ETS domain repressor Aop, is a target of the MAPK pathway in the eye and of the DJNK pathway during dorsal closure in the embryo. In both pathways, Aop acts as a repressor that is inactivated by phosphorylation. The expression of a phosphorylation defective form of Aop prevents photoreceptor cell specification in the eye (Rebay and Rubin 1995), and changes in cell shape in the embryonic lateral epidermis. In the eye, Aop competes with the ETS domain transcriptional activator Pointed P2 (Brunner et al. 1994; O’Neill et al. 1994). Pointed function, however, is not required for dorsal closure because embryos lacking both maternal and zygotic pointed function do not display a dorsal open phenotype and the expression of dpp in the leading edge cells is not altered in these embryos (Mayer & Nüsslein-Volhard 1988; Riesgo-Escovar & Hafen, unpubl.). Therefore, although distinct MAP kinase pathways appear to regulate the same repressor in the eye and in the embryo, they activate different activators, Pnt-P2 in the eye and DJun in the embryo. The regulation of a cellular response by the activity of a MAP kinase cascade involving phosphorylation of two antagonistic transcription factors appears to be a common mechanism for reproducible activation of target genes in response to small changes in the activity of the signaling cascades.

The change in cell shape of the lateral epidermis occurs in two phases (Young et al. 1993). In the first phase, the cells of the leading edge begin to stretch dorsally. In a second phase, the remaining cells ventral to the first row change shape. Interestingly, mutants in components of the DJNK and the Dpp pathway affect these two phases differently. Whereas loss-of-function mutations in either DJNK or DJun block cell shape changes of all cells, mutations in the genes coding for the Dpp receptors tkv and put block only the second phase. In tkv mutants, the leading edge cells stretch but the remaining cells of the lateral ectoderm fail to change shape. It appears, therefore, that Dpp signaling is required for the induction of cell-shape changes in the ectodermal cells located ventral to the leading edge cells. This is supported by our observation that expression of dpp in the leading edge cells is controlled by the DJNK-regulated activities of DJun and Aop.

Is the regulation of dpp expression by DJun and Aop direct? dpp expression during the later stages of embryogenesis, when dorsal closure occurs, has been referred to as phase III expression. An ~500-bp enhancer fragment located 400-bp upstream of the transcription start site is necessary and sufficient for dpp ectodermal phase III expression (Schwyter et al. 1995). DJun and Aop may regu-

Figure 7. tkv mutants exhibit a partial dorsal closure phenotype. (A) Cuticle of a tkv^{207} mutant embryo. Note the centrally located dorsal hole (marked by a white arrow). Unlike bsk and DJun, the most dorsal cuticle is turned inwards and the mouthhooks are retained inside the embryo. The corresponding Cora staining [B] shows that the leading edge cells stretch, but the underlying rows of cells fail to stretch. Two pairs of black arrows mark the dorsal and ventral membranes of two leading edge cells, and the black arrowhead marks cells directly underneath the leading edge cells. (C–I) Expression of dpp or tkv^{35D} suppresses the bsk mutant phenotype. (C–E) and (C–I) sibling embryos from the same experiments, respectively. (C) Df(2L)/flp147E homozygous embryo; (D) UAS-dpp/69B Gal4 embryo; (E) Df(2L)/flp147E; UAS-dpp/69B Gal4 embryo. Note the small dorsal hole (arrow) in (E), and compare with (C). (F) Df(2L)/flp147E/bsk^{1} embryo; (G) UAS-tkv^{35D}/69B Gal4 embryo, and (H) Df(2L)/flp147E/bsk^{1}; UAS-tkv^{35D}/69B Gal4 embryo. In this weaker bsk^{1} mutant combination (compare C with F) expression of tkv^{35D} results in almost complete suppression of the bsk mutant phenotype. Both E and H embryos are dorsalized, as are the D and G controls. (I) bsk^{1}/Df(2L)/flp147E; UAS-tkv^{35D}/69B Gal4 embryo derived from a bsk^{1} germ line. Note partial dorsal closure; this phenotype is very similar to bsk zygotic phenotypes.
Figure 8. Model of the involvement of the DJNK and the Dpp pathway in dorsal closure. [A,B] Prior to activation of DJNK by DJNK activity in the cells of the leading edge, dpp expression is repressed by unphosphorylated Aop protein. Upon activation of DJNK by a yet unknown signal possibly transduced by Dcdc42 (Riesgo-Escovar et al. 1996), DJNK is activated and translocates into the nucleus, where it phosphorylates Aop and DJun. In analogy to phosphorylation by Map kinase (Rebay and Rubin 1995), phosphorylation of Aop by DJNK inactivates DJNK activity, whereas phosphorylation of DJun activates transcription of the target genes dpp and puckered (puc). puc expression in the leading edge cells is regulated by DJNK activity (Riesgo-Escovar et al. 1996). Dpp secreted from the leading edge cells acts as a signal that directly or indirectly induces the ventrally adjacent cells to stretch. [C] In schematic form the changes in cell shape that occur in the lateral epithelium in wild-type embryos, in embryos with mutations in genes coding for components of the DJNK pathway or the Dpp pathway. The leading edge cells are hatched. Whereas mutations blocking the DJNK pathway including mutations in DJun block stretching of all lateral epithelial cells, Dpp pathway mutants do not affect the stretching of the leading edge cells.

late dpp expression in the leading edge cells by directly binding to sites within this enhancer fragment. Indeed, this enhancer fragment contains multiple consensus sites for binding of AP-1 and ETS proteins. In particular, we found one site where an AP-1- and an ETS-binding site are partially overlapping. Further analysis and dissection of this enhancer will be necessary to establish whether, indeed, DJun and Aop regulate dpp expression by directly binding to this dpp enhancer fragment. There is precedent for AP-1-mediated regulation of TGFβ transcription: in humans, the TGF-β1 promoter is positively controlled by AP-1 (Birchenall-Roberts et al. 1990).

The joint requirement of DJun and Dpp function during embryogenesis appears to be unique to dorsal closure, because other processes involving Dpp such as the establishment of dorso-ventral polarity is not affected in DJun mutants. In vertebrates, the Dpp homolog TGF-β and the AP-1 transcription factor act together during wound healing. During this process, TGF-β induces collagenase (matrix metalloprotease-1) gene expression in keratinocytes by activating AP-1 (Mauviel et al. 1996). Collagenases are involved in the breakdown of the fibrillar collagen and are important in a number of physiological processes involving connective tissue remodeling such as embryonic development or tumor metastasis (Woessner 1991). It is likely that the breakdown of the extracellular matrix is an important step in the wound healing process which, like dorsal closure, results in the closing of an epithelial sheath. In analogy, DJun activity may control a Drosophila collagenase that dissolves the extracellular matrix, thereby permitting the cells to stretch laterally. A protein immunologically related to the human type-IV collagenase has been identified in Drosophila and shown to accumulate in invasive tumors (Woodhouse et al. 1994). Furthermore, overexpression of a truncated type IV collagen interferes with dorsal closure (Borchiellini et al. 1996). In DJun mutants, cells may not be able to change shape because they are firmly attached to the basement membrane. Conversely, in tkv mutant embryos the leading edge cells are able to stretch more extremely than in the wild-type. This suggests that in these cells, AP-1 activity is not induced by Dpp but by some other signal, probably from the amnioserosa cells. As in wound healing, Dpp is required to allow more ventrally located cells to change shape, perhaps by inducing AP-1 activity. Although further evidence for the analogy between dorsal closure and wound healing has to await the cloning and characterization of the corresponding collagenase genes, it is an interesting possibility that the connection between the NK and TGF-β signaling pathways has been conserved during evolution. In this respect, it is interesting to note that human TGF-β1 transcription is positively regulated by AP-1 activity (Birchenall-Roberts et al. 1990), and that TGF-β1 autoinduction is also mediated by AP-1 (Kim et al. 1990).

Materials and methods

Genetics

put135, putl1, UAS-tkvQ253D [=tkvQ25D], UAS-dpp, tkvmutl, tkvmutl, a CyO and TM3 balancer chromosomes with y° insertions were obtained from R. Burke, D. Nellen and K. Basler (Universität Zürich, Switzerland). sE-hs promoter constructs of wild-type DJun [DJun], dominant negative DJun [DJunQ25D], or activated Jun [~Jun3 X ~Jun3 ] were from D. Bohmann and M. Mlodzik (European Molecular Biology Laboratory, Heidelberg, Germany). UAS-aop and UAS-aopwere from I. Rebay and G.

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Hypomorphic mutations in \( I(2R)IA109 \)

We prepared stocks that were \( y\- w\- \) doubly balanced in the sec-zygotes \( UAS\-\)construct/69B driver embryos by lack of expression of \( Aop \) in the lateral epithelium during dorsal closure (data not shown). We obtained an SM6B and TM3 balancer chromosomes with \( eve\-\lacZ \) and \( ftz\-\lacZ \) insertions (“blue” balancers) and a deficiency uncovering the \( DJun \) locus, \( D\) (2R) \( X1 \) from the Bloomington Stock Center. \( bsk \) alleles and \( l(2R)IA109 \) were from the Nüsslein-Volhard laboratory. All other alleles and markers are as described (see Lindsley and Zimm 1992, Riesgo-Escovar et al. 1996).

For experiments of \( bsk\- \) homozygotes with \( UAS\-\)tkv\(^{\text{aop}} \) or \( UAS\-\)dpp under the 69B Gal4 driver (Brand and Perrimon 1993), we prepared stocks that were \( y\- w\- \) doubly balanced in the second and third chromosomes over a \( bsk\- \) allele and either a UAS construct or the 69B Gal4 driver in the third chromosome, and crossed flies with the UAS constructs to flies with the Gal4 driver. These experiments were done at 25°C. We used both a set of second and third chromosome balancers with \( \lacZ \) insertions or with \( y\- \) insertions, and distinguished the \( bsk\- \) homozygotes \( UAS\-\)construct/69B driver embryos by lack of \( \lacZ \) staining, or the absence of \( y\- \). Embryos of all the predicted phenotypes were found at the expected ratios.

For experiments with \( aop \) and \( l(2R)IA109 \), \( DJun \) and mammalian \( e\-\hs\-\jun\) constructs, we recombined the mutation or construct on \( bsk\- \) or \( l(2R)IA109 \) chromosomes. In all cases, multiple recombinants were isolated, and more than one recombinant was tested with identical results. To induce \( DJun \) or \( Jun \) expression, 24 hr egg layers were subjected to heat shocks at 37°C for 15, 30, or 60 min. We found that the duration of the heat shock was unimportant (within this range) in all experiments, except for \( e\-\hs\-\DJun^{\text{ben}} \), where 1 hr was required to observe the reported phenotypes. In parallel, \( bsk\- \) embryos carrying the same constructs were heat shocked, and the resulting phenotypes were compared. Expression of \( Jun^{\text{null}} \) in a wild-type embryo by a 1 hr heat shock during germ band extension and retraction may give rise to an extreme phenotype akin to dorsalization: Embryos show only naked cuticle and appeared rounded (data not shown). This is consistent with these embryos having strong overexpression of \( dpp \) (Fig. 6). \( e\-\hs\-\jun^{\text{null}} \) embryos without heat shock also show partial dorsalization. Similar, but weaker, phenotypes were observed in heat shocked \( hs\-\DJun \) embryos. For induction of \( DJun \) mutant clones, \( l(2R)IA109 \) was recombined unto a 42 FRT chromosome (Xu and Rubin 1993). Eye clones were induced by crossing 42 FRT \( l(2R)IA109 \) flies to flies carrying a \( hs\-\flip \) construct and a 42 FRT, \( P[w^\text{+}] \) chromosome. The progeny resulting from this cross was heat shocked to induce \( \flip \)-mediated recombination. Germ-line clones were induced in females of the genotype \( hs\-\flip \), 42 FRT \( l(2R)IA109/42 FRT \) \( \text{ovo}^D \) by 1 hr heat shocks at late third instar larval stage (Chou and Perrimon 1996). These females were mated to \( DJun \) mutant males, or to males carrying a deficiency uncovering \( DJun \). Both crosses gave identical results.

For the \( UAS\-\)aop\(^{\text{WT}}/69B \) Gal4 experiments, crosses were done at 25°C and at 29°C. In parallel, we examined \( UAS\-\)aop\(^{\text{null}}/69B \) driver. The resulting embryos were wild-type. We used both a second and a third chromosome insertions of both types of \( UAS\-\)aop constructs. As a control for \( aop \) expression and for the 69B Gal4 driver, \( UAS\-\)aop\(^{\text{WT}}/69B \) Gal4 embryos were stained with Aop antibody. Staining was seen in all ectodermal cells at germ band extension/retraction and dorsal closure stages (data not shown).

Histology

Embryo X-Gal stainings and antibody stainings were done as described previously in Riesgo-Escovar et al. (1996), except that Cora antibody (Fehon et al. 1994) was used at a 1:2000 dilution. Cora protein expression is normal in \( bsk \) (Riesgo-Escovar et al. 1996), \( tkv \), \( put \), and \( aop \) mutants (Figs. 1, 3, 7). Aop antibody, a kind gift of I. Rebay and G. Rubin, was used at a 1:10 dilution. Anti 8-Gal antibody from Cappel was used at a 1:2000 dilution. Tubulin stainings were according to Foe (1989). Cuticle preparations were done as reported in Riesgo-Escovar et al. (1996). Digitized images were assembled by use of Adobe Photoshop 3.0 software.

Kinase assays

Recombinant AopA was purified from bacteria as described (Brunner et al. 1994), and kinase assays were done and quantified as described (Riesgo-Escovar et al. 1996). Recombinant GST \( \text{Kinase assays} \)

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